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THE UTILIZATION OF THE CALCIUM OF CARROTS BY ADULTS [1]

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INTRODUCTION

Although vegetables, because of their mineral content, have been recognized these many years as a valuable adjunct to man's dietary, only recently has it been pointed out (Mitchell and Curzon, '39) that, on a dry matter basis, some of the commonly-used vegetables are superior to milk as a source of calcium. In view of the current trend toward a more wide-spread and generous usage of vegetables, the question arises as to the extent to which these foods can satisfy man's need for calcium. Blatherwick and Long ('22) tried to find the answer to this question by using a mixture of vegetables; likewise, Sherman and Hawley ('22), in their attempt to determine whether or not "children could utilize calcium in the form of vegetables as efficiently as they had utilized the calcium of milk," fed more than one vegetable simultaneously. The purpose of the present work, however, was to obtain a quantitative measure of the availability of the calcium of a specific vegetable, since any data obtained on a mixture would be applicable only to a combination of those constituents in their original proportions.

[1] These data were reported, in brief, before the American Institute of Nutrition at the annual meeting in New Orleans on March 13, 1940.

[2] A portion of these data was submitted by Miss Esther J. Rutherford in partial fulfillment of the requirements for the degree of Master of Science at the University of Illinois, June, 1940.

In the selection of the vegetable to be studied, consideration was given to native foods which were commonly and frequently used and which were readily available, from the standpoint of both supply and cost, throughout the year. Accordingly, the carrot was chosen since it fulfilled all these qualifications and enjoyed the added characteristic of being adaptable to various ways of serving. Moreover, the carrot was one of the foods comprising the basal dietary used in earlier studies (Kinsman, Sheldon, Jensen, Bernds, Outhouse and Mitchell, '39) from which the authors concluded that children on low calcium diets do not exhibit a maintenance requirement for calcium. This postulation, it was believed, could be valid only if the calcium of the basal food were no better utilized than the milk calcium. Naturally, any information on the availability of the calcium of the various constituents of that dietary would aid in settling the question.

EXPERIMENTAL

The experiment reported here is part of an investigation of the ability of seven adults to utilize dietary calcium; the first paper concerning that research dealt with milk calcium (Breiter, Mills, Dwight, McKey, Armstrong and Outhouse, '41). The basal period (metabolic periods 8 through 13) of the milk study also served as the basal period for the present study; it followed the period during which carrots were ingested (metabolic periods 1 through 7) and was preceded by a 7-day interval during which the subjects ingested large quantities of calcium in an otherwise unrestricted dietary. Throughout both the basal and the carrot periods, the basal dietary and the vitamin-carrying supplements were identical with those fed in the study on milk.

During the period in which the carrot calcium was being tested, all subjects received equal quantities of carrots; [3] i.e.,

[3] California-grown carrots, because of their higher calcium content and their superior palatability, were used instead of the local varieties which were already in winter storage; 400 gm., either cooked or raw, were fed. The remaining 300 gm. were consumed in the form of an unadulterated pulp-juice mixture (S & W brand) which had the same calcium content (i.e., 29 mg. per 100 gm.) as did the raw young carrot.

700 gm. daily. Such rations of a bulky food may seem unduly large, but they contained just enough calcium to give a reliable test in this type of procedure. Although the carrots furnished 202 mg. of calcium, the difference between the average calcium intakes during the carrot and basal periods was only 179 mg.; this discrepancy was due to an augmentation of the intake of basal calcium by an unanticipated increase in the consumption of bread following the withdrawal of the carrots.

RESULTS

The subjects were in good condition, both physically and mentally, throughout the experiment. Even though a temporary abdominal distress followed the ingestion of the large quantities of raw or cooked carrots, this discomfort passed off within a few hours, and all subjects had good appetites for the succeeding meal. The large amount of bulk resulted in a greater volume of fecal material and an easier evacuation of the intestine, but the time required for the passage of the carmine (which was given for the purpose of marking off the periods) was not uniformly affected; it was shortened for one subject, lengthened for four, and unchanged for the other two. A yellow pigmentation of the skin, which gradually increased in intensity and persisted for several weeks after the carrots had been withdrawn from the dietary, was noted early in the experiment. All subjects maintained their customary body weights throughout the study.

The data obtained during the carrot period are recorded in table 1 as the average daily values involved in the exchange of calcium. Inasmuch as the data for the basal period have already been reported (Breiter et al., '41), they will not be repeated in detail here. During the 35 days that carrots were eaten, the mean calcium intakes averaged 449 mg., and the daily net losses of calcium by subjects Jd, Bm, Hb, Mh, Jo, Ws and Rd, respectively, were 34, 72, 58, 74, 58, 49 and 121 mg.

During the basal period the amount of calcium ingested by the subjects averaged 270 mg., and the means of the negative balances were 67, 88, 80, 67, 120, 64 and 141 mg. in the above

TABLE 1

Daily calcium exchange and the utilization of carrot calcium.

PERIOD	INTAKE	OUTPUT Urinary	OUTPUT Fecal	BALANCE	INTAKE	OUTPUT Urinary	OUTPUT Fecal	BALANCE
	Subject Jd		Weight in kg. 53		Subject Bm		Weight in kg. 55	
	Age 22	Sex F	Height in cm. 166		Age 22	Sex F	Height in cm. 167	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Carrot 1	376	198	240	—62	383	276	238	—131
2	429	194	276	—41	440	311	188	— 59
3	446	225	285	—64	456	295	233	— 72
4	464	252	231	—19	465	269	231	— 35
5	484	235	238	+11	494	299	276	— 81
6	433	211	238	—16	436	276	243	— 83
7	419	218	251	—50	434	250	229	— 45
Ave.	436	219	251	—34	444	282	234	— 72
Basal ave.	248	179	136	—67	264	227	125	— 88
% utilization	$\frac{67-34}{436-248} \times 100 = 17.6$				$\frac{88-72}{444-264} \times 100 = 8.9$			
	Subject Hb		Weight in kg. 60		Subject Mh		Weight in kg. 61	
	Age 27	Sex F	Height in cm. 161		Age 24	Sex M	Height in cm. 166	
Carrot 1	388	134	323	—69	402	135	367	—100
2	437	155	343	—61	436	134	511	—209
3	456	151	371	—66	474	149	349	— 24
4	474	128	416	—70	483	155	370	— 42
5	494	143	395	—44	504	155	424	— 75
6	458	155	335	—32	468	132	339	— 3
7	434	154	343	—63	459	137	389	— 67
Ave.	449	146	361	—58	461	142	393	— 74
Basal ave.	278	141	217	—80	274	122	219	— 67
% utilization	$\frac{80-58}{449-278} \times 100 = 12.9$				$\frac{67-74}{461-274} \times 100 = 0.0$			
	Subject Jo		Weight in kg 64		Subject Ws		Weight in kg. 65	
	Age 42	Sex F	Height in cm. 161		Age 22	Sex M	Height in cm. 171	
Carrot 1	343	180	261	—98	401	90	330	— 19
2	407	173	282	—48	455	103	311	+ 41
3	422	182	262	—22	473	111	512	—150
4	437	220	216	+ 1	500	136	457	— 93
5	469	201	358	—90	533	136	435	— 38
6	421	190	309	—78	483	134	365	— 16
7	411	207	279	—75	464	124	409	— 69
Ave.	416	193	281	—58	473	119	403	— 49
Basal ave.	231	193	158	—120	309	127	247	— 64
% utilization	$\frac{120-58}{416-231} \times 100 = 33.5$				$\frac{64-49}{473-309} \times 100 = 9.2$			
	Subject Rd		Weight in kg. 74					
	Age 24	Sex M	Height in cm. 181					
Carrot 1	392	125	409	—142				
2	460	129	431	—100				
3	468	139	462	—133				
4	491	148	463	—120				
5	512	172	368	— 28				
6	474	149	448	—123				
7	461	175	484	—198				
Ave.	465	148	438	—121				
Basal ave.	289	142	288	—141				
% utilization	$\frac{141-121}{465-289} \times 100 = 11.4$							

order. When these values are compared, according to the formula for computing percentage utilization (Breiter, Mills, Dwight, McKey, Armstrong and Outhouse, '41), with the individual intakes and the balances occurring in the carrot period, the following values for per cent utilization of carrot calcium are obtained: 17.6, 8.9, 12.9, 0.0, 33.5, 9.2 and 11.4. The mean of the values for the seven subjects is 13.4%.

DISCUSSION

The values reported here for the utilization of carrot calcium range from 0 to 34%. This range is great, and no tenable explanation presents itself for the fact that subject Mh made no use whatsoever of the carrot calcium whereas subject Jo utilized fully one-third of it. (Neither age nor sex difference could explain the variation.) On the other hand, the utilization figures for the five other subjects do not deviate greatly from the average; they range from 9 to 18%. In view of the fact that the values for the seven subjects do range from 0 to 34%, one well might question the worth of that 13.4% average in predicting the extent to which any one individual can utilize the calcium of carrots.

A comparison of these data and those obtained for these same subjects in a previous study on the availability of the calcium of milk (Breiter, Mills, Dwight, McKey, Armstrong and Outhouse, '41) indicates that the calcium of carrots was, as an average, only about one-half (55%) as available as was the calcium of milk. But the individual data are not consistent: only two (Ws and Rd) of the subjects approximated the average; three fell much lower; and two (Jo and Jd) showed virtually equal utilization with both sources. The values obtained for the utilization of the calcium of carrots and that of milk were, respectively, 18 and 15% for Jd, 9 and 31% for Bm, 13 and 35% for Hb, 0 and 18% for Mh, 34 and 30% for Jo, 9 and 20% for Ws and 11 and 20% for Rd. If one were to draw a general conclusion from this comparison, one would be inclined to state that adults probably do not utilize the calcium of carrots any more (and many undoubtedly even less) efficiently than they do that of milk.

In searching for the cause of the apparent inferiority in the availability of the carrot calcium, as demonstrated by the five subjects of the present study, one finds that the calcium of the two foods was not fed under strictly comparable conditions—i.e., the carrots were rich in "indigestible" carbohydrate,[4] whereas the milk was free of this substance. If fiber is capable of inhibiting calcium utilization, it could have been responsible for the low values observed in this study inasmuch as such large quantities were eaten. However, the effect could not have been brought about by a hastening of the passage of the fecal mass through the intestinal tract, since Jd was the only one of the seven subjects showing a decrease in the time required for the appearance of carmine, and she utilized carrot calcium better than she did milk calcium. Moreover, the literature reveals neither conclusive nor consistent evidence that the presence of fiber in the diet affects adversely the utilization of calcium.

None of the previously published calcium metabolism data obtained on human subjects ingesting carrots can be computed in terms of percentage utilization. There are studies, however, in which a comparison of the availability of the calcium of this food is made with that of milk. The studies of Sherman and Hawley ('22) and of Edelstein ('32), both of which used children as subjects, indicate poorer calcium retention when carrots were fed than when milk was the chief source of calcium. However, the data of Sherman and Hawley do not lend themselves to accurate evaluation with respect to the availability of the calcium of carrots since a mixture of spinach, celery and carrots was fed, and the poor calcium retention observed may have been due to the unavailability of the spinach calcium. Although one of Edelstein's infant subjects retained more calcium when fed carrots than he did when a "milchgriessbrei" constituted the main source of calcium, another child did better on the milk diet. The results of Rose

[4] Dr. T. S. Hamilton of the Animal Husbandry Department generously made analyses on the carrots and reported the following values based on wet weight: crude fibre, 0.99%; cellulose, 1.36%; and lignin, 1.59%.

('20) show the same lack of consistency—i.e., one of the women apparently made as good use of carrot calcium as she did of milk calcium, whereas the other woman retained more calcium when milk was fed. Obviously, none of this work on human beings gives substantial evidence concerning the comparative value of these two foods as source of calcium.

There are, however, two animal studies in which the observed data are overwhelmingly in favor of milk. One of these is the McClugage and Mendel ('18) study on two dogs; Shields, Fairbanks, Berryman and Mitchell ('40) on recalculating those data found that dogs A and B, respectively, utilized 52 and 40% of the calcium of milk but only 24 and 29% of the calcium of carrots—utilization only 60% as efficient as that found for milk calcium. The other study was carried out by the latter group of authors on twenty-two pairs of rats. Everyone of the rats receiving carrots retained less calcium than did its pair-mate which received an equivalent amount of calcium as milk; the mean value indicating only 85% as good usage. These data on animals not only indicate more efficient utilization of milk calcium over carrot calcium but also show greater consistency than those observed on man.

The carrot has ranked high as a source of dietary calcium ever since Rose ('20) reported that the calcium needs of man could be met "largely, if not wholly, from carrots." The facts demonstrated by the present study indicate that, for the subjects used here, the reverse is true. All of the subjects were in negative balance to a marked degree even though they were consuming as much as 700 gm. of carrots daily. On the basis of their individual abilities to utilize carrot calcium, these subjects would have had to consume an average of over 2 kg. of carrots daily in order to be in calcium equilibrium. Under practical dietary conditions, a 100 gm. portion would be considered an average-sized serving, and 200 gm. a generous one, but the latter quantity would add only 60 mg. of calcium to the intake, and, from this, the subject who had the highest rate of utilization could have secured only 20 mg.—a small fraction of the requirement. Why a discrepancy should exist

between the data of Rose and those herein reported is not clear. Obviously, the carrots used in Rose's study were richer in calcium than were the California-grown variety used in this study,[5] but this difference cannot be entirely responsible for the difference in the extent to which carrots met the requirement for calcium in the two experiments. A more likely explanation might be found in the state of calcium nutrition of the two groups of subjects. The fact that three of Rose's subjects, when ingesting no more than 383 mg. calcium daily, not only attained calcium equilibrium but even stored calcium would suggest that their tissues were not well saturated with calcium and were, perhaps, avid for it since the quantity of urinary calcium they excreted was very small. If Rottensten's study on rats ('38) is applicable to man and if Rose's subjects really were poorly nourished with respect to calcium, then one would suspect them of utilizing their calcium very efficiently. Such superior utilization would have made possible the satisfaction of their requirements with much smaller quantities of carrots than would have been needed by the seven subjects of the present study or by the nine men (assuming for them utilization rates for carrot calcium no greater than those found for milk) studied by Steggerda and Mitchell ('41). Whether or not many individuals exist who can do as well as did Rose's subjects is not known. One thing is clear however; if the adults of this study were to satisfy their calcium needs through the ingestion of this vegetable, they would have to eat such appalling quantities of it that they would have little digestive capacity for those other foods which are considered essential for health. It seems justifiable, therefore, to brand the carrot an unimportant source of calcium for these people.

SUMMARY

The extent to which the calcium of carrots could be utilized by adults was determined for four women and three men. Following the procedure previously used in this laboratory,

[5] See footnote 3, page 2.

two levels of calcium were fed, one averaging 270 mg. daily, the other 449. The same basal dietary was fed throughout the experiment, the higher level having been brought about by the daily ingestion of 700 gm. carrots, the total calcium content of which averaged 202 mg. The basal period lasted for 34 days and the carrot period for 39 days. On the basis of differences between the intakes of these two periods and differences in the magnitude of the negative balances, the amount of carrot calcium utilized was computed; the values obtained were 17.6, 8.9, 12.9, 0.0, 33.5, 9.2 and 11.4%, averaging 13.4. Five of these seven subjects showed poorer utilization of the carrot calcium than they did of milk calcium in a previous study.

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THE OCCURRENCE OF FREE AND BOUND BIOTIN[1]

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ONE FIGURE

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Biotin has recently assumed a position of great interest and importance in animal nutrition. du Vigneaud, Melville, György and Rose ('40) and György, Rose, Hofmann, Melville and du Vigneaud ('40) reported that it is identical with vitamin H, the factor curative of egg white injury in rats and chicks. It has been demonstrated by Williams and co-workers (Eakin, McKinley and Williams, '40; Eakin, Snell and Williams, '40; and György, Rose, Eakin, Snell and Williams, '41) that the biotin of the diet is fixed by an albumin fraction of raw egg white, "avidin", thus preventing the absorption of biotin from the intestinal tract and causing an induced deficiency. Biotin will also combine with "avidin" in vitro (Eakin, Snell and Williams, '40).

Hegsted, Oleson, Mills, Elvehjem and Hart ('40) observed in chicks a scaly dermatitis which was not due to pantothenic acid deficiency but was cured by concentrates of vitamin H. Hegsted, Mills, Briggs, Elvehjem and Hart ('41) have since found crystalline biotin to be completely curative. György

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and Poling ('40) state that biotin is connected with the prevention of graying in rats and mice and Nielsen and Elvehjem ('41) have shown that biotin is involved in the spectacle eye syndrome of the rat.

McHenry and Gavin ('41) showed that beef liver fractions fed to rats caused acute fatty livers, the formation of which could not be prevented by administration of choline. Several biotin concentrates had a similar effect, hence biotin was indicated as the active factor. This effect demonstrates an important role for biotin in the synthesis of lipids by the rat.

In view of the growing significance of biotin in animal nutrition, additional information concerning its distribution and the forms in which it occurs in various materials would be highly desirable.

Snell, Eakin and Williams ('40) showed that biotin occurs, at least in liver, in a bound insoluble form, from which it could be freed by autolysis. We have already reported that acid hydrolysis of liver and kidney caused large increases in the amount of extractable biotin. In this paper we report the occurrence of bound biotin in a number of other tissues and its liberation by various means and present values for the biotin content of these tissues.

EXPERIMENTAL RESULTS

Assay method

Biotin was determined by a microbiological method (Peterson, McDaniel and McCoy, '40) employing Cl. butylicum no. 21 (Wisconsin). This organism on a basal medium of 2% glucose, 0.1% asparagine and 0.11% salts (K_2HPO_4, 10 gm.; KH_2PO_4, 10 gm.; $MgSO_4 \cdot 7\,H_2O$, 4 gm.; NaCl, 0.2 gm.; $MnSO_4 \cdot 4\,H_2O$, 0.2 gm.; $FeSO_4 \cdot 7\,H_2O$, 0.2 gm.) requires only the addition of biotin for maximum growth (Snell and Williams, '39). The measure of growth is the turbidity as read in an Evelyn photoelectric colorimeter. The organism requires anaerobic conditions for growth.

The stock culture is carried on sterile soil and a loopful is inoculated into a tube of the basal medium plus 0.25%

peptone. After 18–20 hours incubation, 10 cc. of this culture is centrifuged and resuspended in 10 cc. of sterile, freshly-boiled water. Three drops of this washed culture are added to each tube.

Lipless test tubes (25 × 200 mm.) are graduated at 36 cc. for the determination. A small amount of reduced iron is placed in each tube to aid in maintaining anaerobic conditions. The biotin solution for the standard curve or an extract of the material to be assayed, dissolved in 10 cc. or less, is placed in the tube and the volume brought to 11 cc. with water.

The basal medium is prepared in such concentration that addition of 25 cc. of it to each tube, i.e., to the 36 cc. mark, will give the desired concentrations. The pH of this basal medium should be 6.6–6.7 before addition. The tubes are plugged and autoclaved 30 minutes at 15 pounds pressure. They are cooled quickly and inoculated immediately. An oat-jar is prepared by filling a suitable container one-fourth full with oats, and adding just enough water to cover the oats. The assay tubes are placed in the jar and a glass top sealed on with plasticine. The respiration of the oats removes the oxygen and establishes a partial pressure of carbon dioxide. The jar is then placed in the incubator at 37°C. for 3 days.

After incubation the tubes are removed, shaken well and their turbidity read against the uninoculated basal medium set at 100. With excess biotin a reading of 15–20 is obtained. One-half of the difference between the inoculated control and the maximum reading, arbitrarily fixed as 20, is taken as half-maximum growth. A unit is defined as the quantity of material per cubic centimeter of medium which is required to give half-maximum growth. This unit is 0.000,010 micrograms (0.01 millimicrograms, or 0.01 mγ) for pure biotin, and by comparison of the unit weights, the biotin content of crude materials may readily be calculated. For example, if a tube containing the extract of 20 μg. of yeast per cubic centimeter gave half-maximum growth, the yeast contained 500 mγ of biotin per gram of dry substance.

As far as we have been able to determine, the organism is able to use any water-soluble biotin; that is, hydrolysis of such complex materials as molasses, peptone, and a liver autolysate caused no increase in the available biotin. It has become evident that there is considerable variation between duplicates even on pure biotin. The accuracy of the method is $\pm 20\%$.

A number of problems have been encountered in the use of the assay. If the pH of the basal medium before addition is brought to 6.3, a flocculent precipitate appears during autoclaving and incubation. This difficulty can be avoided by holding closely to a pH of 6.6–6.7. Care must be taken to prevent contamination of the culture. Whenever it seems to have occurred, growth is always maximum, i.e., the contaminants appear to have synthesized enough biotin to meet the requirements of the assay organism, although as judged by microscopic examination, the cells in such tubes consist mostly of the usual rods of *Cl. butylicum*. In an effort to avoid the cumbersome oat-jars and reduced iron, sodium thioglycollate was tested for maintenance of proper anaerobic conditions. The optimum concentration was 0.000,1 M, but even with this, many tubes failed to initiate growth. The addition of agar, cystine, or sodium hydrosulfite was also unsatisfactory.

Liberation of biotin

Detailed studies were made of the conditions required for the freeing of biotin from yeast and liver. Figure 1 shows one experiment with these two materials. The acid hydrolysis was done by autoclaving with 4N H_2SO_4. With liver the amount of water-soluble biotin reached a maximum at 1 hour and up to 4 hours no destruction was noted. A series was also run with 7N H_2SO_4. Here too, 1 hour was sufficient for complete liberation, and 4 hours treatment caused no destruction. With yeast 1 hour with 4N H_2SO_4 gave a maximum value but after 2–3 hours some destruction began.

Autolysis liberated biotin much more readily from yeast than from liver. Twenty-four hours autolysis, either with

toluene alone or with toluene and chloroform, freed 80–90% of the biotin of the yeast. The maximum was reached in 6 days and no destruction occurred during 3 weeks. No inhibition by chloroform of the freeing of biotin during autolysis as experienced by György ('39) was observed.

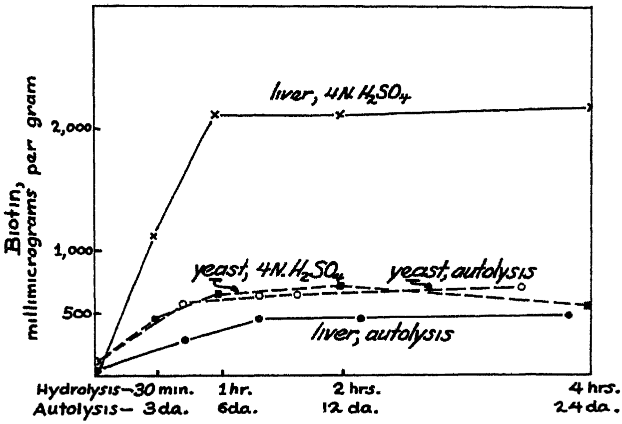

Fig. 1 Freeing of biotin in liver and yeast.

The release of the biotin from liver by autolysis was very slow and incomplete. Never more than 30% was rendered soluble by this method. Chloroform had no effect on the rate of liberation. The autolysis curve in figure 1 was run both with toluene alone and with chloroform and toluene and the values were identical.

The biotin content of samples of liver [2] is given in table 1. Autolysis again failed to free all of the biotin. Treatment with pepsin for 7 days at 50°C. also gave incomplete liberation. Other lengths of time and concentrations of pepsin were tested, but all were less effective than the example cited. Hydrolysis with trypsin for 3 hours at 50°C. completely liberated the biotin. Boiling of the liver to destroy the enzymes cut down the yield of biotin as did the use of a lower temperature. A hydrolysis with acid was run in our laboratories as a check and gave a figure in agreement with the maximum.

[2] Treated in various ways by the Difco Laboratories, Inc.

TABLE 1

Liberation of biotin from liver by various treatments.

TREATMENT	CONDITIONS	BIOTIN
		mγ/gm.
Autoclaved with water	1 hour, 15 pounds	60
Autolysis, in presence of chloroform and toluene	7 days, 37°C., pH 6.0–6.5	550
Pepsin	7 days, 50°C., pH 3.0	460
Trypsin	50°C., 3 hours	2,200
,,	50°C., 3 hours (liver boiled first)	1,700
,,	37°C., 7 days	1,800
,,	37°C., 7 days, fresh trypsin added daily	2,100
H_2SO_4	4N, 1 hour, 15 pounds	2,140

Assays of biological materials

In table 2 are assays of the biotin content of a variety of natural materials illustrating the occurrence of free and bound biotin. The water extracts were made by suspending 1 gm. of the substance, homogenized by the method of Potter and Elvehjem ('36), in 50 cc. of water and autoclaving for 30 minutes. The suspension was adjusted to 50 cc., filtered, and the filtrate was properly diluted for assay. The acid extractions were carried out by autoclaving 1 gm. of the homogenized material with 50 cc. of 2N H_2SO_4 for 2 hours, and subsequently removing the $SO_4^=$ with $Ba(OH)_2$. From the work on liver and yeast, extraction with 4N H_2SO_4 for 1 hour seemed to be satisfactory, but because of occasional destruction of biotin with the 4N acid, 2N H_2SO_4 was subsequently adopted. Although maximal values were obtained under the conditions outlined, other conditions of extraction might give higher values.

Large increases, up to sixtyfold, were observed on hydrolysis of meats. Yeast, liver, kidney, egg yolk, and pancreas are all high in biotin, but lean meats are relatively low in the factor. Cow manure is rich in biotin but these samples do not approach the value of 150,000 mγ per gram given by Robbins and Schmidt ('39). Urine was assayed by addition

of the whole material to the medium. Preliminary hydrolysis of the urine did not increase the values given. In confirmation of the report of Snell, Eakin and Williams ('40), the biotin content of egg yolk is not increased by hydrolysis.

TABLE 2

Assays of natural materials.

MATERIAL	BIOTIN CONTENT, $M\gamma$/GM. DRY MATTER: Water extract	BIOTIN CONTENT, $M\gamma$/GM. DRY MATTER: Acid extract
Yeast and animal products		
Baker's yeast	27	700 (4N)
Brewer's yeast	100	830 ('')
Beef kidney	120	2500 ('')
Beef, lean	20	130 ('')
Cheese	26	24 ('')
Cow manure (winter ration)	500	490 ('')
Cow manure (pasture)	1700	1250 ('')
Egg white	530	480 ('')
Egg yolk (defatted)	1000	870 ('')
Heart	—	190 ('')
Milk	30–40 $m\gamma$/cc.	30–40 $m\gamma$/cc. (1N)
Meat scrap meal	12	48 (4N)
Pork kidney	100	4000 ('')
Pork liver	33	2000 ('')
Pork, lean	35	60 ('')
Pancreas	670	1230 (2N)
Urine (cow)	17 $m\gamma$/cc.	—
'' (human)	3.0 $m\gamma$/cc.	—
Vegetables and other green plant materials		
Alfalfa	260	280 (4N)
Beets	35	27 (2N)
Beans, string	200	230 ('')
Cabbage	62	32 (4N)
Carrots	400	285 (2N)
"Cerophyll" (2 samples)	290–450	330–440 (4N)
Peas, fresh	95	110 (2N)
Potato	17	17 ('')
Spinach	450	480 (4N)
Tomato	1000	400 (2N)
Fruits		
Apple	43	60 (2N)
Banana	182	105 ('')
Orange	100	80 ('')
Peach	440	400 ('')
Raspberry	590	460 ('')
Seeds and products		
Barley	180	200 (4N)
Beans, navy	30	64 ('')
Corn (sample #1)	77	80 ('')
'' (sample #2)	50	50 ('')
'' (sample #3)	360	230 ('')
Corn steep	700	—
Oats (sample #1)	83	240 ('')
'' (sample #2)	71	170 ('')
Rice polish	170	270 (2N)
Rye	23	57 (4N)
Soybeans (sample #1)	200	540 (4N)
'' (sample #2)	83	830 ('')
'' (sample #3)	100	400 ('')
Wheat (sample #1)	22	67 ('')
'' (sample #2)	113	70 ('')
Wheat bran	83	140 (2N)
White flour	—	5.2 (2N)
Rice bran concentrate (Vitab)	1300 2000	— —
Nuts		
Peanut (roasted)	110	400 (4N)
'' (raw)	170	210 (2N) 200 (4N)
'' (raw)	110	210 (4N)
Pecan	80	114 (2N) 270 (4N)
Walnut, English	140	370 (2N) 280 (4N)

Assay of milk samples required special study. Addition of whole milk is impossible because of the precipitation of casein during fermentation. Repeated precipitation and washing of the casein gave a filtrate containing 98% of the biotin, but this was too tedious for a routine method. Hydrolysis of the milk for 1 hour with 1N H_2SO_4 gave a maximal value. A biotin recovery was run by adding 0.128 μg. of biotin to the milk before hydrolysis. Recovery was 104%. The simplest method, and the one finally chosen, was to dilute 5 cc. of milk to 50 cc. with an acetate buffer of pH 4.6. The casein was filtered off through a dry filter paper. This filtrate gave values equal to those by acid hydrolysis. The range of values was 30–40 μg./cc.

The biotin of green plants and vegetables exists in a free, water-soluble form. Some destruction by acid was noted with tomato. Alfalfa, string beans, carrots, spinach, and grass powders[3] are fair sources, while beets, cabbage, peas, and potatoes are low. The biotin of fruits is also in a water-extractable form. Peaches and raspberries rate high in this group, in contrast to apples and oranges which are low.

The picture in regard to seed products is extremely confused. Soybeans, navy beans, oats and rye appear to contain combined biotin, but barley and corn gave no increase on hydrolysis. The values for wheat are contradictory. Preliminary extractions with ether or chloroform, in order to permit better subsequent penetration by the acid, resulted in no improvement. The best method of preparation of grain samples for assay is still uncertain. A cereal concentrate[4] proved to be an excellent source of biotin.

The occurrence of firmly bound, water-insoluble biotin in nuts seems definitely established. Preliminary ether or chloroform extractions again yielded no improvement. Values of 200–400 mγ per gram were obtained for all of the samples.

[3] Cerophyll.

[4] Vitab. Rice Bran Concentrate.

Destruction of biotin by acid

During treatment of crude materials with 4N H_2SO_4, destruction of biotin has been frequently observed. None has been found with 1N acid and only a few cases with 2N. Evidence of partial destruction was mentioned in the discussion of yeast and low values with acid were noted for cow manure, tomatoes, bananas and nuts. Table 3 shows experiments regarding the destruction of biotin in three different materials. Destruction with acid was not confined to any single type of material, some of the samples being high in protein, some in carbohydrate, and others in lipids. However, it was evident that a considerably longer time was required to produce any destruction in yeast and animal tissues.

TABLE 3

Acid destruction of biotin.

SAMPLE	BIOTIN (mγ/GM. DRY MATTER)				
	Time of acid treatment				
	None	30 min.	1 hour	2 hours	4 hours
Molasses concentrate, 4N H_2SO_4	2640	2200	—	820	720
Corn + 1460 mγ of added biotin, 4N H_2SO_4	1540	1540	—	1000	840
Dried juice of young barley					
1N H_2SO_4	570	480	500		
2N H_2SO_4		370	280		
4N H_2SO_4			25		

SUMMARY

The biotin of yeast and animal products occurs primarily in a water-insoluble firmly-bound combination. Increases of from two- to sixtyfold occurred on hydrolysis. Vegetables, green plant materials, and fruits contain a water-extractable form, while seeds and nuts appear to have considerable amounts of bound biotin.

The biotin may be freed either by hydrolysis with acid, 2N H_2SO_4 for 2 hours being the generally optimal treatment,

or with enzymes such as trypsin. Autolysis is excellent for liberating the biotin from yeast.

Under treatment with 4N H_2SO_4, destruction of biotin has been found in yeast, corn, barley-juice powder, and soybeans.

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STUDIES IN NICOTINIC ACID METABOLISM

I. THE FATE OF NICOTINIC ACID IN MAN [1]

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A large number of publications have appeared within the last 3 years (reviewed by Lepkovsky, '40, and Morgan, '41) presenting quantitative data on the excretion of "nicotinic acid" in the urine of man and animals under a variety of conditions. Obviously, the figures and their interpretation depend almost entirely upon the specificity of the given methods and upon the relative yield of color reacting substances from techniques employed in the splitting or the transformation of the various nicotinic acid derivatives into nicotinic acid itself.

In considering the question of specificity, pyridine and its derivatives, including nicotine in the urine of smokers, demand attention. In the majority of reports in which the original König color reaction with CNBr and aniline was used, the relatively high daily outputs of 10 to 30 mg. of "nicotinic acid" in human urine could be traced to the presence of variable amounts of nicotine and of other pyridine derivatives in the urine.

[1] The data in this article are taken from a thesis to be presented by Herbert P. Sarett in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of Arts and Sciences of Duke University.

A preliminary report was made by the authors before the meeting of the American Society of Biological Chemists at Chicago, April, 1941.

[2] Upjohn Fellow in Biochemistry.

Of the known nicotinic acid derivatives, only nicotinuric acid and trigonelline have been well established as constituents of human and dog urine (Ackermann, '12; Linneweh and Reinwein, '32 a and '32 b; Perlzweig, Levy and Sarett, '40; Melnick, Robinson and Field, '40). Euler and Schlenk ('39) and H. Kohn and P. Handler (personal communication) have found the concentration of coenzyme in urine to be below the sensitivities of their methods. Calculated from Kohn's data, not more than 4% of the acid hydrolyzable nicotinic acid derivatives could be in the form of coenzyme. Our own studies indicate that free nicotinic acid and nicotinamide could account for only 25 to 40% of the acid hydrolyzable nicotinic acid derivatives in normal urine, and even a smaller proportion after the administration of relatively large doses of nicotinic acid or amide (see below). The two derivatives which predominate in human and in dog urine, both normally and after the administration of either nicotinic acid or the amide, are nicotinuric acid and trigonelline. Trigonelline, which constitutes over 90% of the measurable nicotinic acid derivatives in normal urine (the remainder comprising the acid hydrolyzable fraction) also accounts for 60 to 95% of these derivatives after the administration of either nicotinic acid or the amide. Although the acid hydrolyzable nicotinic acid derivatives make up a small fraction of the total excretion in normal urine, over one-half of this fraction, as judged from the data in table 2, appeared to be in the form of nicotinuric acid. This is contrary to the statement of Melnick, Robinson and Field ('40) that no nicotinuric acid is found in normal human urine.

Recognizing these facts, all techniques and methods employed for the determination of nicotinic acid and its derivatives in urine must be so designed and the conditions so defined as to ensure the quantitative measurement of all of these nicotinic acid derivatives and not of any extraneous pyridine compounds. Since with these color reactions nicotinamide and nicotinuric acid yield variable intensities of the same color as obtained with nicotinic acid, they must first be completely

hydrolyzed to nicotinic acid by boiling with strong acid. Trigonelline can only be measured quantitatively after a strong alkali-urea hydrolysis following the acid hydrolysis.

Furthermore, since trigonelline is a normal constituent of a number of plant tissues and foods, notably coffee and the leguminous seeds (Maier-Bode and Altpeter, '34), the intake of these in the diet must either be excluded or controlled in metabolism experiments.

In the investigations reported in this and in the subsequent communication (Sarett, '42) we have been guided by the above considerations, while being fully aware of the fact that the measurement of the nicotinic acid derivatives mentioned above may not yet yield a complete picture of the fate of nicotinic acid in the human and animal organisms, as will be apparent from the presented data.

This communication deals with a study of the fate of nicotinic acid in normal man under controlled dietary conditions, before and after the administration of various nicotinic acid derivatives.

EXPERIMENTAL

The experiments reported here are divided into three parts. One series consists of the experiments performed on three normal individuals (the authors) who were maintained on a rigorously controlled diet consisting of trigonelline free foodstuffs. Levels of daily excretion of nicotinic acid and trigonelline on this diet and after oral doses of nicotinic acid derivatives were determined (table 1).

The second series of experiments was a set of analyses of the urines of thirty-three medical students, who were on various diets low in trigonelline, and of three fasting students (table 3).

The third series was a set of saturation tests with nicotinamide on six normal young adults (table 4).

All urines analyzed were 24-hour specimens which were collected under toluene and kept in the refrigerator. The acid hydrolyzable nicotinic acid fraction (NA) and trigonelline

were determined by the previously described method (Perlzweig, Levy and Sarett, '40). Since publication it has undergone the following minor modification and improvement:

After boiling a suitable urine sample [3] with 10 cc. of 7 *N* HCl and 0.25 cc. of concentrated HNO_3 over a microburner for 1 hour, there is very little charring and the pigments are largely decolorized. The nitric acid, however, produces a light yellow color in urine which is not removed by the subsequent procedures. This difficulty is overcome by extracting the acid digests, in 25 ml. glass stoppered graduated cylinders, with two or three 5 cc. portions of ether. The ether portions, containing most of the yellow pigments, are pipetted off; the ether remaining in solution in the digests is removed by immersing the cylinders into a beaker of warm (40–50°C.) water; after cooling, the solutions are adjusted to a convenient volume in the cylinders. This extraction with ether results in excellently low blank values in the final stage of the colorimetric determination. The ether extraction was shown not to cause any losses of nicotinic acid or of trigonelline. The rest of the analytical procedure as previously described remains unaltered.[4]

NA and trigonelline excretion after administration of nicotinic acid and derivatives

On a low trigonelline diet consisting of meats, white bread, rice, corn, milk, milk products and fruits, three subjects excreted 1.1 to 1.5 mg. of NA per day and 9 to 13 mg. of trigonelline per day (table 1).

[3] In the presence of relatively large amounts of nicotinuric acid, correspondingly smaller aliquots of urine were taken and the boiling continued for 90 minutes. If the nicotinuric acid is not completely hydrolyzed to nicotinic acid by the acid digestion, it will be split in the alkali urea hydrolysis of trigonelline, and will be erroneously measured as trigonelline.

[4] The most uniformly reproducible results in the final color reaction were obtained by using a 3% solution of crystalline CNBr (Eastman). This solution keeps well in dark amber glass in the refrigerator for at least 3 weeks and possibly longer.

TABLE 1

Excretion of NA and trigonelline by three subjects, on a "trigonelline free" diet, after ingested doses of nicotinic acid compounds.

	DAYS	SUBJECT H NON-SMOKER			SUBJECT S MODERATE SMOKER			SUBJECT P HEAVY SMOKER		
		NA [1]	Trig.	Total [2]	NA [1]	Trig.	Total [2]	NA [1]	Trig.	Total [2]
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Control	1	1.4	12.2	12.4	1.1	13.0	12.8	1.4	12.0	12.2
	2	1.2	9.0	9.3	1.5	12.2	12.7	1.4	11.0	11.3
After 200 mg. dose of nicotinic acid	3	13.5	27.9	38.6	6.9	39.3	42.3	21.0	38.3	55.5
	4	1.0	19.1	18.2	1.2	18.2	17.6			
After 200 mg. dose of nicotinamide	9	1.3	51.0	47.3	1.6	68.0	62.6	2.0	45.0	42.5
	10	1.4	22.7	21.8	1.1	24.5	23.1	1.5	17.8	17.5
After 200 mg. dose of trigonelline	11	1.2	181.0		1.2	170.0		1.9	196.0	
	12	1.0	18.5		1.1	19.4		1.4	26.0	

[1] NA includes all nicotinic acid derivatives hydrolyzable by boiling with 7 N HCl.

[2] Total represents the total excretion of nicotinic acid and trigonelline, calculated in terms of nicotinic acid.

These data were substantiated by the values of 8 to 12.6 mg. of trigonelline excreted per day by three individuals after 3 days of starvation (table 3) and show that the dietary trigonelline was reduced to a minimum. The figures for NA given here and in the other tables represent $HCl–HNO_3$ acid hydrolyzable derivatives of nicotinic acid as described above, and include the nicotinic acid, nicotinamide, coenzyme and nicotinuric acid which may be present in the urine.

The daily dietary nicotinic acid intake of the three subjects was kept at a fairly constant level of about 30 to 40 mg.[5] Test doses of nicotinic acid derivatives were equally divided between breakfast and lunch and were taken orally at the end of these meals.

Two hundred milligram doses of nicotinic acid, taken on the third day (table 1) resulted in the added excretion of 35

[5] An average daily diet included 300 gm. of meats containing about 60 to 70 µg. NA per gram, and 2 kg. of cereals, breads and vegetables, which contained roughly about 8 to 10 µg. NA per gram.

to 44 mg. of total NA and trigonelline in the next 2 days.[6] The average for the days of the control period served as a base line for these calculations. This added excretion represented only 17 to 22% of the ingested dose. Of the total extra excretion 55 to 84% was in the form of trigonelline. The remaining 16 to 45% was entirely nicotinuric acid. This was demonstrated in the following manner.

Nicotinuric acid was completely hydrolyzed by the 7 *N* HCl–HNO_3 mixture which was used for nicotinic acid determinations. After treatment of nicotinuric acid in a water bath for 1 hour with 1 *N* HCl, the Bandier color reagents (Bandier and Hald, '39) gave only 35% of the color of the equivalent amount of nicotinic acid. Nicotinamide, on the other hand, was completely hydrolyzed by 1 *N* HCl or the 7 *N* HCl–HNO_3 treatment. Two equations have been set up:

$$\text{Nicotinuric acid} + \text{other nicotinic acid derivatives} = 7\ N\ \text{HCl value}$$
$$0.35(\text{nicotinuric acid}) + \text{other nicotinic acid derivatives} = 1\ N\ \text{HCl value}$$

Upon subtraction there was obtained the equation:

$$0.65\ (\text{nicotinuric acid}) = 7\ N\ \text{HCl value} - 1\ N\ \text{HCl value}$$

Typical figures for the partition of the NA fraction of normal urines and those obtained after doses of nicotinic acid are given in table 2.

TABLE 2

Partition of NA fraction in normal urines and after dose of nicotinic acid, by 1 N HCl and 7 N HCl hydrolyses. Explanation in text.

	NICOTINIC ACID			NICOTINURIC ACID
	After 1 *N* HCl	After 7 *N* HCl	As nicotinuric acid	
	µg./cc.	*µg./cc.*	*µg./cc.*	*%*
Normal urines	0.7	1.4	1.1	78
	0.9	1.5	0.9	60
	0.9	1.8	1.4	78
Urines after dose of nicotinic acid	13.0	41.0	43.0	105
	26.4	72.0	70.0	97
	7.2	19.2	18.4	96

[6] It is to be noted that while the NA fractions returned to a normal level on the second day after each dose of nicotinic acid or the amide, the trigonelline was more slowly excreted, maintaining a higher level in the urine for several days.

Since nicotinuric acid is the glycine conjugate of nicotinic acid, and since trigonelline is a methylated derivative of nicotinic acid, the effects of administrations of 2 gm. of glycine and of 400 mg. of choline in combination with 200 mg. nicotinic acid on the relative proportions of urinary trigonelline and nicotinuric acid were tried. No demonstrable effects of these additions were observed in the partition of the nicotinuric acid and trigonelline fractions in the urine in all three subjects. Likewise upon repeating these experiments in dogs (Sarett, '42) but using much larger amounts of glycine and choline no effect was observed.

Of the 200 mg. of nicotinamide taken on the ninth day, 18 to 30% could be accounted for by the added trigonelline excretion of the following 2 days. Distinctly different from the excretion after a dose of nicotinic acid was the lack of any increase in the NA fraction. To show that the question of absorption of nicotinic acid or nicotinamide from the gastro-intestinal tract did not control these results, nicotinamide was given intravenously, with the same resultant excretion as that obtained after the oral dose.

Trigonelline ingested in 200 mg. doses was almost completely excreted (80–90%) in the following 2 days. Nicotinuric acid taken orally in 100 mg. doses by the three subjects was excreted, in unchanged form, to the extent of 56, 60 and 65%, with no concomitant increase in the trigonelline fraction. The utilization of nicotinuric acid by man thus appears to be doubtful, but because of its relative insolubility the complete absorption from the gastrointestinal tract is questionable. A more definite solution is being sought by means of parenteral administration of nicotinuric acid.[7]

Levels of nicotinic acid and trigonelline excretion of thirty-three medical students

In the course of their metabolic experiments on the effects of various dietary regimes upon the composition of the urine,

[7] Since this paper has gone to press it has been determined that nicotinuric acid given intravenously in 100 mg. doses to three subjects was excreted to the extent of 96, 94 and 97%.

thirty-three medical students who did not drink coffee were asked to abstain from leguminous foods. These regimes included normal, high protein, high carbohydrate-low protein diets, high water intake and starvation. The range and averages of NA and trigonelline excretions are shown in table 3.

TABLE 3

Daily excretion of NA and trigonelline by young normal adults on low trigonelline diet and in starvation.

	NA		TRIG.	
	Range	Average	Range	Average
	mg.	*mg.*	*mg.*	*mg.*
Various diets				
33 subjects (see text)	0.7–2.2	1.2	5–25.4	15.1
Starvation—Subject 1		1.1		9.3
Subject 2		2.0		12.6
Subject 3		1.4		8.2

The diets of these students could not be as rigorously controlled as were the diets of the first three subjects, and this resulted in several (seven) cases of high levels of trigonelline excretion. The average trigonelline excretion, omitting these questionable values was 15.1 mg. per day. The average for the more rigorously controlled diet (table 1) was 11 mg. trigonelline per day. The difference was due to the diet. The 8 to 12.6 mg. of trigonelline (average, 10 mg.) excreted by three individuals on the third day of fasting was an excellent check of the validity of the above values.

In this group of thirty-three students the NA excretion ranged from 0.7 to 2.2 mg. per day with an average daily excretion of 1.2 mg. Normal urines analyzed by our method have always given values in this range, in smokers as well as non-smokers. In the six habitual smokers in this group the NA values ranged from 1.0–2.2 mg., average 1.2; the trigonelline values ranged from 10.4 to 15.5, average 12.8 mg. These values do not differ in any significant manner from those obtained on the group as a whole.

The 24-hour urine of a 7-week-old premature baby weighing 3.1 kg., who was on a trigonelline free diet of milk and lactose, contained 0.14 mg. NA and 2.5 mg. of trigonelline.

On the basis of body weight the levels of NA and trigonelline excretion were much higher than those in the adult.

Saturation tests with nicotinamide

In all of the above experiments test doses of nicotinic acid and nicotinamide had been administered to normal individuals in a good nutritional state. Of the 200 mg. of nicotinic acid or amide ingested only 12 to 45% (average 22%) was accounted for by the added urinary NA and trigonelline. There were wide individual variations.

The ingested nicotinic acid which could not be recovered in the urine either went through another metabolic pathway or was taken up by the tissues which were not fully saturated. In dogs, which had been saturated with nicotinic acid, all of the extra administered nicotinic acid could be recovered in the urinary NA and trigonelline (Sarett, '42). In man this has not been possible to achieve thus far, as shown in the following experiment.

Six medical students were given 500 mg. of nicotinamide orally for 6 successive days. They abstained from trigonelline containing foods during this time. The urines of the first and sixth days were collected and analyzed. The results are summarized in table 4. The control urines were the normal urines collected in the preceding part of the experiment.

TABLE 4

Saturation tests on six individuals receiving 500 mg. nicotinamide per day for 6 days on low trigonelline diet.

SUBJECT NUMBER	CONTROL URINE			URINE OF FIRST DAY AFTER 500 MG. NICOTINAMIDE				URINE ON SIXTH DAY			
	NA [1]	Trig.	Total [1]	NA [1]	Trig.	Total [1]	Per cent recovery [2]	NA [1]	Trig.	Total [1]	Per cent recovery [2]
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	%	*mg.*	*mg.*	*mg.*	%
32 B	0.8	12.8	12.3	5.4	105	100	18	7.3	182	171	32
32 C	0.9	8.5	8.5	10.0	104	104	19	8.0	233	218	42
14	1.2	16.4	15.9	4.9	103	98	17	4.6	163	151	28
40	2.2	12.5	13.4	4.2	110	103	18	7.6	164	155	28
48	1.1	39.4	36.5	10.3	142	138	21	11.8	240	228	40
50	1.6	20.7	20.2	1.0	50	46	5	6.4	169	158	27

[1] See table 1.

[2] Per cent recovery refers to the portion of the 500 mg. dose of nicotinamide accounted for by the extra total excretion of NA and trigonelline.

In all cases, both on the first and sixth days, trigonelline comprised over 90% of the added total excretion. Of the remaining NA fraction only 65 to 80% was nicotinuric acid. This differs from the results obtained after the administration of nicotinic acid, in which the NA fraction was proportionately larger and was composed entirely of nicotinuric acid.

On the first day 17 to 21% of the ingested dose could be accounted for in the urines of five of the subjects, but only 5% in the urine of subject no. 50. On the sixth day, it was possible to account for 27 to 42% of the dose in this way.[8] Although this showed an increase in nicotinic acid saturation of the individuals, it also indicated that another pathway for nicotinic acid metabolism may exist in man.

The small output of NA and trigonelline by subject 50 after the first dose of 500 mg. of nicotinamide indicated the possibility of a deficiency in nicotinic acid. This was tested by giving 500 mg. of nicotinamide to ten more normal people all of whom responded with an excretion of NA and trigonelline of at least 17 to 20% of the test dose. In nutritionally deficient hospital patients, however, only a small portion (0 to 5%) of the nicotinamide could thus be accounted for, and in some of these patients several large doses of nicotinamide were necessary before any increase in NA and trigonelline could be detected.

Further results obtained on thirty unselected patients upon admission to the hospital indicate that it is possible to establish a test on this basis as a means for detection of nicotinic acid deficiency in man. This work is now in progress.

SUMMARY

Trigonelline and nicotinuric acid are the two known end products of nicotinic acid metabolism found in human urine.

For a study of normal trigonelline excretion in man, a diet free of coffee, peas, beans and other legumes is necessary. On

[8] After the daily intake of 500 mg. nicotinamide for 30 days, three normal subjects excreted no more than 40% of the ingested dose.

such a diet, rigorously controlled, three individuals excreted 9 to 13 mg. of trigonelline per day. On less rigorously controlled diets, twenty-six students excreted an average of 15 mg. of trigonelline per day. Three individuals, after 3 days of fasting, excreted 8 to 12.6 mg. trigonelline per day.

The normal daily excretion of acid hydrolyzable nicotinic acid derivatives (NA) by thirty-six subjects on different diets and under fasting conditions was 0.7 to 2.2 mg. This level was not affected by smoking.

There is a constant loss of at least 10 mg. of known nicotinic acid derivatives in the urine daily, regardless of diet.

Of 200 mg. oral doses of nicotinic acid, 25 to 90 mg. could be accounted for by the increase in urinary trigonelline and nicotinuric acid. Trigonelline accounted for 55 to 84% of this increase. The remainder was nicotinuric acid. The feeding of glycine and choline with 200 mg. doses of nicotinic acid did not change the proportions of trigonelline and nicotinuric acid excreted.

Of 200 mg. doses of nicotinamide, 36 to 60 mg. could be accounted for by the increase in urinary trigonelline. There was no increased excretion in the acid hydrolyzable nicotinic acid fractions.

Trigonelline is not utilized by man when taken orally, and is excreted almost completely as such. Nicotinuric acid when ingested is excreted to the extent of about 60%, unchanged, and without an accompanying increase in trigonelline.[9]

After six daily doses of 500 mg. of nicotinamide the urinary excretion of NA and trigonelline in six subjects accounted for 27 to 42% of the ingested dose, compared to 5 to 21% which could be accounted for after the first day. Over 90% of this added excretion was in the form of trigonelline. Of the remaining NA fraction, 65 to 80% was nicotinuric acid.

The extra excretion of NA and trigonelline after a test dose of nicotinamide may serve as an indication of the nutritional status of an individual with respect to nicotinic acid.

[9] See footnote 7, page 29.

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STUDIES IN NICOTINIC ACID METABOLISM

II. THE FATE OF NICOTINIC ACID IN NORMAL AND BLACK TONGUE DOGS [1]

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WITH THE TECHNICAL ASSISTANCE OF MOLLY STENHOUSE

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There are many similarities in man and dog with respect to the need for nicotinic acid and the fate of this substance. The avitaminotic diseases, pellagra and black tongue, which result from the deficiency of nicotinic acid have many like characteristics. Both man and dog can form nicotinuric acid and trigonelline from nicotinic acid (Ackermann, '12; Sarett, Huff and Perlzweig, '42; Melnick, Robinson and Field, '40).

This communication deals with a study of the nicotinic acid and trigonelline excretion of dogs both at different levels of nicotinic acid intake and in black tongue. Since urinary trigonelline may be attributed both to nicotinic acid metabolism and to the ingestion of many foods containing trigonelline, it was necessary to carry out these experiments with a trigonelline free diet (Sarett, Huff and Perlzweig, '42; Perlzweig, Levy and Sarett, '40).

The level of trigonelline excretion drops markedly after a few weeks on a black tongue producing diet, and practically disappears with the onset of black tongue. More striking

[1] The data in this article are taken from a thesis to be presented by Herbert P. Sarett in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of Arts and Sciences of Duke University.

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than the difference in level of trigonelline excretion, however, is the difference between the complete retention of large doses of nicotinic acid by the black tongue dog and the complete excretion of this compound by the saturated animal. Most of this excretion is in the form of trigonelline, as already reported for man (Sarett, Huff and Perlzweig, '42).

EXPERIMENTAL

Full grown dogs (10–14 kg.) were kept in individual metabolism cages and the urines were collected daily under toluene and analyzed for nicotinic acid and trigonelline by the previously described method (Sarett, Huff and Perlzweig, '42; Perlzweig, Levy and Sarett, '40). The only modification of the method was the extraction of the acid hydrolysate with small amounts of ether (5 cc.) to help insure low blanks. This was particularly advisable when the nicotinic acid and trigonelline content of the urine became so low that it was necessary to take 40 to 50 cc. of urine for analysis.

For the low nicotinic acid diet, the black tongue producing ration of Koehn and Elvehjem ('37) was used, since we were unable to detect trigonelline in either the casein or the corn meal. It is possible that very small amounts of trigonelline are contained in these foods, but this would not be enough to make a significant difference either in the figures or their interpretation. Crude casein was substituted for the purified casein, since the nicotinic acid content of the crude product was only about 2 μg. per gram. The yellow corn meal contained about 8 μg. nicotinic acid per gram. The percentage composition of the diet was as follows: yellow corn meal 72, casein (crude) 18, cottonseed oil 5, cod liver oil 2, calcium carbonate 1, calcium biphosphate 1, sodium chloride 1. This mixture was cooked with four parts of water, so that each kilogram of cooked food contained 200 gm. of this mixture. On this basis, the nicotinic acid content of the diet was 1.2 mg. per kilogram. The dogs received weighed amounts of the food corresponding to approximately 1 kg. cooked food per 10 kg. body weight or 0.1 to 0.2 mg. nicotinic acid per kilogram body weight. A supple-

ment of 2 mg. of riboflavin and 2 mg. of thiamine chloride was given each dog every week.

Excretion on a low nicotinic acid diet

Three healthy dogs were fasted for 4 days before receiving the low nicotinic acid diet. At the end of this time they excreted 0.3 to 0.7 mg. NA and 3.3 to 7.8 mg. trigonelline per day (table 1). The low nicotinic acid diet was fed for 20 days,

TABLE 1

Daily urinary excretion of NA and trigonelline of three dogs on a diet low in nicotinic acid.

DAY OF DIET	A — 14.8 KG.			B — 10.7 KG.			C — 11.2 KG.		
	NA [1]	Trig.	Total [2]	NA [1]	Trig.	Total [2]	NA [1]	Trig.	Total [2]
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
After 4 days starvation	0.7	7.6	7.5	0.5	7.8	7.5	0.3	3.3	3.3
1–5	0.7	2.6	3.0	0.6	2.5	2.8	0.6	3.2	3.5
18–20	0.6	1.6	2.0	0.5	2.7	2.9	0.5	1.1	1.5
	25 mg. nicotinic acid orally on twenty-first day								
21	1.2	3.1	4.0	1.5	8.1	8.8	0.8	1.6	2.2
22	1.1	2.8	3.6	0.7	3.5	3.8	0.4	0.8	1.1
	25 mg. nicotinic acid intravenously on twenty-fourth day								
24	2.7	6.1	8.2	1.2	4.8	4.3	0.8	1.8	2.4
25	1.0	4.4	5.0	0.9	5.5	5.8	0.6	1.4	1.9
	25 mg. nicotinamide intravenously on twenty-seventh day								
27	3.9	9.8	12.7	1.3	10.6	10.8	0.6	6.4	6.4
28	1.6	8.1	8.9	0.9	6.4	6.7	0.6	2.2	2.6

[1] NA includes all nicotinic acid derivatives hydrolyzable by boiling with 7 *N* HCl.

[2] Total represents the sum of NA and trigonelline calculated in terms of nicotinic acid.

during which time the dogs continued to eat approximately the same amount of food per day and increased their weights almost back to the pre-fasting level. During these 20 days the NA excretion of the dogs changed very little, but the trigonelline dropped to 1.1 to 2.7 mg. per day.

An oral dose of 25 mg. of nicotinic acid at this time was almost completely retained by each of the three dogs. Of the

25 mg., 18.2 to 24.3 mg. were retained, as estimated by the following 2 days' excretion. The excretion during the eighteenth to twentieth days serves as a baseline for the calculations. A second 25 mg. dose of nicotinic acid was given intravenously 3 days later, and here the dogs retained 16.0 to 23.8 mg. Three days later a third dose of 25 mg. nicotinamide was substituted for the nicotinic acid and in this case only 7.4 to 19.2 mg. of the dose was retained. The greater part of the amount which was excreted was trigonelline. Most of the extra excretion in the NA portion was in the form of nicotinuric acid.

Although it was logical to expect a greater retention of nicotinic acid after the first oral dose, the question of efficient absorption could not be overlooked. To investigate this, the feces were analyzed and showed no differences in NA and trigonelline content, either before or after an oral dose of nicotinic acid was given.

The feces were collected at frequent intervals and kept in 5 N HCl. The specimens for the eighteenth to twentieth days inclusive were pooled to give a basal sample, and those passed during the 3-day period following the oral dose of 25 mg. nicotinic acid likewise pooled and examined for any increases. The feces were homogenized and analyzed for NA and trigonelline in a manner similar to that used for urine.

The fecal NA of the dogs on the basal diet was 0.6 to 1.6 mg. per day and the trigonelline only 0.15 to 0.3 mg. per day. After the 25 mg. oral dose the NA was 0.7 to 1.1 mg. per day and the trigonelline 0.1 to 0.3 mg. per day. Analyses of feces of these dogs on a high nicotinic acid diet (1.1 mg. per kilogram) gave similar results. Most likely this fecal NA and trigonelline was entirely of bacterial origin.

Excretion on high nicotinic acid diet

For the high nicotinic acid diet, 10 mg. of nicotinic acid were added to each kilogram of cooked food. This was checked by analysis of the cooked food. Thus each dog eating 1 kg.

of food per 10 kg. body weight was ingesting approximately 1.1 mg. nicotinic acid per kilogram per day.

The three dogs used in the low nicotinic acid experiment became the subjects in this experiment immediately after the third 25 mg. dose of nicotinamide. Another dog (D) which had been on the same low diet for other experiments was also included. After 9 days on this diet there was a rapid increase in trigonelline excretion and very little change in NA excretion (table 2). On the sixth to ninth days the dogs

TABLE 2

Daily urinary excretion of NA and trigonelline of four dogs on a diet high in nicotinic acid (1.1 mg. per kg. body weight).

DAY OF DIET	A — 14.5 KG.			B — 10 KG.			C — 11.1 KG.			D — 11.8 KG.		
	NA [1]	Trig.	Total [2]	NA [1]	Trig.	Total [2]	NA [1]	Trig.	Total [2]	NA [1]	Trig.	Total [2]
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1–3	1.1	5.2	5.8	0.4	1.1	1.4	0.5	2.7	2.9	0.4	1.5	1.7
4–6	1.4	5.1	6.0	0.5	2.6	2.8	0.5	4.9	4.9	0.4	4.3	4.3
7–9	1.6	7.3	6.2	0.5	4.9	4.9	0.6	5.0	5.1	0.5	4.1	4.2
10	25 mg. nicotinic acid intravenously											
10	5.7	29.3	32.1	1.6	22.7	22.0	2.8	19.8	20.6	1.0	23.6	22.2
11	1.9	15.7	16.0	0.4	8.7	8.2	0.6	11.8	11.2	0.4	11.3	10.6
12	1.2	7.1	7.6	0.6	7.0	6.9	0.5	6.9	6.7	0.4	6.8	6.5
13	100 mg. nicotinic acid intravenously											
13	17.1	43.2	56.0	17.8	84.5	93.8	19.7	70.0	82.7	5.3	53.8	53.7
14	7.5	63.0	64.1	1.0	26.9	25.2	1.1	21.7	20.6	1.3	58.0	53.5
15	1.8	16.2	16.4	0.5	8.9	8.5	0.5	10.9	10.3	0.4	10.5	9.9
16	1.4	13.1	13.2	0.4	5.5	5.4	0.5	9.3	8.8	0.4	7.2	6.9

[1] NA includes all nicotinic acid derivatives hydrolyzable by boiling with 7 *N* HCl.

[2] Total represents the sum of NA and trigonelline calculated in terms of nicotinic acid.

excreted a total of 4.2 to 8.2 mg. in the form of NA and trigonelline on an intake of 12 to 15 mg. per day.

At this time a 25 mg. intravenous dose of nicotinic acid was almost completely excreted; dog A apparently retained none and the others retained about 5 to 8 mg. It was impossible to calculate this retention accurately since the basal excretion varied somewhat. However, for the purpose of these calculations we arbitrarily took the twelfth day as the basal

level of excretion for the dogs on this diet. It is to be remembered that these dogs retained 5 to 10 mg. of the nicotinic acid received in the diet each day, and that the retention and excretion calculated in all of these figures were only for the extra doses of nicotinic acid or nicotinamide.

On this basis the added excretion of total NA and trigonelline (calculated as nicotinic acid) after the first 100 mg. intravenous dose of nicotinic acid was 112, 115, 93 and 96 mg. A second 100 mg. dose given 3 days later resulted in the same amount of added excretion. After receiving intravenous doses of 100 mg. of nicotinamide there were excreted 103, 108, and 105 mg. extra of total NA and trigonelline. These dogs had reached the saturation point. They no longer retained any part of a large dose of nicotinic acid or nicotinamide, but converted all of it into two products—trigonelline and nicotinuric acid. In our experiments on humans (Sarett, Huff and Perlzweig, '42) after repeated dosing with nicotinamide only 27 to 42% of the dose could be accounted for in the urine. There may be another pathway of nicotinic acid metabolism in the human which differs from that in the dogs.

In the urine of dogs trigonelline made up 75 to 94% of this extra excretion when either nicotinic acid or nicotinamide was given. When nicotinic acid was given the 6 to 25% which was excreted in the NA fraction was practically all in the form of nicotinuric acid. However, when nicotinamide was given, only 75 to 85% of this fraction was nicotinuric acid.

In an attempt to influence the proportions of trigonelline and nicotinuric acid formed, choline and glycine, in separate experiments, were added to the diet. Fifteen grams of glycine fed to each of two dogs (B and C) during 3 days preceding an intravenous dose of 100 mg. of nicotinic acid resulted in the same distribution of the NA and trigonelline fractions in the urine as that shown for these dogs in table 2 after a similar dose of nicotinic acid alone. Similarly 4 gm. of choline fed to each of these dogs during 2 days preceding another dose of nicotinic acid did not affect the partition of these fractions in

the urine. Thus the ingestion of large amounts of glycine and choline appears to have no appreciable effect upon the excretion of nicotinuric acid and of trigonelline, respectively.

Excretion of nicotinuric acid and trigonelline

Nicotinuric acid and trigonelline are the main excretory products derived from nicotinic acid. Therefore, in order to investigate whether they pass through the body completely and unchanged, they were administered separately to dogs on the low nicotinic acid diet. Each of three dogs received 100 mg. of trigonelline orally. Within 2 days, 75% had been excreted in the urine unchanged. In the next 2 days 10 to 15 mg. more of trigonelline could be accounted for. Two other dogs received subcutaneously 225 mg. nicotinuric acid (= 150 mg. nicotinic acid) and in 1 day excreted 95% of the nicotinuric acid as such. Since these dogs had been on a low nicotinic acid diet for 5 weeks, this complete excretion of nicotinuric acid showed that the dogs could not utilize nicotinuric acid in place of nicotinic acid, nor could they split it to form nicotinic acid. This is in harmony with the results of Dann and Handler ('41 c), who were unable to cure canine black tongue with nicotinuric acid given subcutaneously, and contrary to the finding of Woolley, Strong, Madden and Elvehjem ('38) in a single case of canine black tongue.

Tissue analysis of dogs on high nicotinic acid diet

We are grateful to Drs. W. J. Dann and P. Handler for analyses of the liver, kidney and muscle of dogs B, C and D, which were performed by their method ('41 a) as soon as the urine experiments on the high nicotinic acid diet were completed.

For normal dogs Dann and Handler ('41 a) found the following values (in micrograms per gram) for the nicotinic acid content of tissue: liver 90–233, mean 153; kidney 75–117, mean 95; muscle 59–80, mean 71. In the tissues of the three dogs saturated with nicotinic acid the following values were

25 mg., 18.2 to 24.3 mg. were retained, as estimated by the following 2 days' excretion. The excretion during the eighteenth to twentieth days serves as a baseline for the calculations. A second 25 mg. dose of nicotinic acid was given intravenously 3 days later, and here the dogs retained 16.0 to 23.8 mg. Three days later a third dose of 25 mg. nicotinamide was substituted for the nicotinic acid and in this case only 7.4 to 19.2 mg. of the dose was retained. The greater part of the amount which was excreted was trigonelline. Most of the extra excretion in the NA portion was in the form of nicotinuric acid.

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For the high nicotinic acid diet, 10 mg. of nicotinic acid were added to each kilogram of cooked food. This was checked by analysis of the cooked food. Thus each dog eating 1 kg.

of food per 10 kg. body weight was ingesting approximately 1.1 mg. nicotinic acid per kilogram per day.

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Daily urinary excretion of NA and trigonelline of four dogs on a diet high in nicotinic acid (1.1 mg. per kg. body weight).

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	NA [1]	Trig.	Total [2]	NA [1]	Trig.	Total [2]	NA [1]	Trig.	Total [2]	NA [1]	Trig.	Total [2]
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1–3	1.1	5.2	5.8	0.4	1.1	1.4	0.5	2.7	2.9	0.4	1.5	1.7
4–6	1.4	5.1	6.0	0.5	2.6	2.8	0.5	4.9	4.9	0.4	4.3	4.3
7–9	1.6	7.3	6.2	0.5	4.9	4.9	0.6	5.0	5.1	0.5	4.1	4.2
10	25 mg. nicotinic acid intravenously											
10	5.7	29.3	32.1	1.6	22.7	22.0	2.8	19.8	20.6	1.0	23.6	22.2
11	1.9	15.7	16.0	0.4	8.7	8.2	0.6	11.8	11.2	0.4	11.3	10.6
12	1.2	7.1	7.6	0.6	7.0	6.9	0.5	6.9	6.7	0.4	6.8	6.5
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13	17.1	43.2	56.0	17.8	84.5	93.8	19.7	70.0	82.7	5.3	53.8	53.7
14	7.5	63.0	64.1	1.0	26.9	25.2	1.1	21.7	20.6	1.3	58.0	53.5
15	1.8	16.2	16.4	0.5	8.9	8.5	0.5	10.9	10.3	0.4	10.5	9.9
16	1.4	13.1	13.2	0.4	5.5	5.4	0.5	9.3	8.8	0.4	7.2	6.9

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For normal dogs Dann and Handler ('41 a) found the following values (in micrograms per gram) for the nicotinic acid content of tissue: liver 90–233, mean 153; kidney 75–117, mean 95; muscle 59–80, mean 71. In the tissues of the three dogs saturated with nicotinic acid the following values were

obtained: liver 150, 178, 159; kidney 93, 93, 68; muscle 85, 88, 91 μg. per gram. Thus it was only in the muscles of these three animals that the nicotinic acid content was found to be above the normal range in each case, while the values for the liver and kidney fell well within that range, and indeed close to the normal means for these tissues. Since the muscles constitute the most extensive of the body tissues, the possibility of storage of nicotinic acid in them may be of some significance.

Black tongue dog

It is usually difficult to follow the urinary excretion of a dog exhibiting black tongue, since the onset of severe symptoms and of anuria is sudden. In some dogs, however, the progress of the disease is more gradual. One such dog of our series developed the mouth lesions and heavy salivation typical of the black tongue dog after 7 weeks on the low nicotinic acid diet. The dog remained moderately sick for almost 3 weeks during which time the daily excretion of NA and trigonelline was measured as shown in table 3. He suddenly became very

TABLE 3

Excretion of NA and trigonelline by a dog during the onset of black tongue and during dosage with nicotinic acid.

DAY	NA	TRIG.	DAY	NA	TRIG.
	mg.	*mg.*		*mg.*	*mg.*
50 [1]	0.4	1.9	71–72 [3]	1.5	0.5
51–52	0.4	1.6	73	1.9	1.1
53	0.6	1.8	74	1.1	2.7
54–55	0.5	0.7	75	1.4	7.4
56–57	0.7	0.8	76	2.9	19.4
58–59	0.5	0.7	77	1.2	6.6
60–61	0.9	0.6	78	1.3	15.9
62	0.6	0.6	79	1.0	9.3
63–64	0.4	0.3	80	1.1	15.3
65–66	0.4	0.2	81	0.8	15.6
67–68	0.4	0.1			
69–70 [2]	0.5	0.1			

[1] Mouth lesions and heavy salivation appeared in dog and persisted for the following 3 weeks.

[2] Dog became very sick. Severe necrosis and bloody diarrhea developed.

[3] Started to receive 25 mg. nicotinic acid subcutaneously each day.

ill on the seventieth day of the diet and was given 25 mg. of nicotinic acid subcutaneously each day, and the urinary NA and trigonelline determined as he recovered.

While the dog was sick, the NA excretion fell very little, but the trigonelline almost disappeared. The figures of 0.1 to 0.2 mg. of trigonelline reported in the table are too low to be reliable and may fall within the limits of error of the analytical method.

With the daily administration of 25 mg. nicotinic acid there was practically no increase in excretion of trigonelline until the fifth day. It then rose slowly and reached a level of about 15 mg. per day. During this period the excretion of NA did not rise significantly. The total nicotinic acid excretion, predominantly as trigonelline, resembled at the end that of normal dogs on a high nicotinic acid intake. The dog used or destroyed 10 mg. of nicotinic acid per day, which is similar to the 7 to 8 mg. per day unaccounted for on the high nicotinic acid diet, shown in table 2.

DISCUSSION

The evidence presented above definitely indicates the importance of trigonelline formation as an index of nicotinic acid metabolism in the dog. After a few days of starvation, there is a small drop in the trigonelline excretion of a healthy dog as the animal begins to conserve its body tissues. While on the low nicotinic acid diet, the trigonelline excretion continues to drop markedly, although NA excretion falls very little. When the dog develops black tongue, the urinary trigonelline disappears almost completely, and it is this cessation of trigonelline excretion which appears at the same time as the severe symptoms of black tongue. This could be due to lack of ability to make trigonelline or to lack of excess of nicotinic acid as the precursor. Since the ability to form trigonelline is recovered in a few days by administering nicotinic acid, it seems that the deficiency is only in the amount of nicotinic acid. However, the nicotinic acid content of tissues of black tongue dogs (Dann and Handler, '41 b) is not far enough below the normal to explain this unless

we assume that the larger part of the nicotinic acid is needed to carry on the functions of coenzyme as we know them now, and that a smaller part is necessary for an unknown, vital step in the body's total metabolism.

After 20 days on a low nicotinic acid diet, three doses of 25 mg. of nicotinic acid were required before the dogs excreted any substantial part of the 25 mg. dose. In the same way, the black tongue dog required 125 mg. of nicotinic acid before any of it was excreted, and it was only after a few more 25 mg. doses, that the proportion of the dose excreted resembled that of a normal dog. This response to dosage with nicotinic acid is the most accurate way of determining whether a dog is totally or partially deficient in nicotinic acid.

SUMMARY

A study of the excretion of acid hydrolyzable nicotinic acid derivatives (NA) and trigonelline in dogs, at different levels of nicotinic acid intake, is presented. Trigonelline and nicotinuric acid are the main end products of nicotinic acid metabolism found in dog urine.

The urinary excretion of trigonelline falls to 1.1 to 2.7 mg. per day on a low nicotinic acid diet, and to 0.1 mg. in the black tongue dog.

On the low nicotinic acid diet or in black tongue several 25 mg. doses of nicotinic acid are required before the dog will excrete part of the dose.

On a high nicotinic acid diet (1.1 mg. per kilogram) dogs excrete a total of about 7 mg. of the 12 to 15 mg. they ingest daily. Over 90% of this is in the form of trigonelline.

After saturation with nicotinic acid, doses of 100 mg. of this compound or nicotinamide are completely excreted in the form of trigonelline (75 to 94%) and nicotinuric acid (6 to 25%). The addition of glycine and choline does not influence the proportions excreted as nicotinuric acid or as trigonelline.

Neither trigonelline nor nicotinuric acid can be utilized by the dog.

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PANTOTHENIC ACID IN THE NUTRITION OF THE RAT[1]

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Lepkovsky et al. ('36) concluded that rats needed a "filtrate factor" which was distinct from what we recognize today as thiamine, riboflavin and pyridoxine. This filtrate fraction has been studied in relation to both the growth of rats and the prevention of graying in pigmented rats. Several earlier investigators (Hartwell, '23; Bakke et al., '30; and Gorter, '35) reported the production of hair depigmentation of black and piebald rats maintained on certain diets. Lunde and Kringstad ('38) and Morgan et al. ('38) observed both graying and retarded growth in black rats reared on synthetic diets. Oleson, Woolley and Elvehjem ('39) and Subbarow and Hitchings ('39) obtained growth responses by rats on adding pantothenic acid concentrates to synthetic rations similar to those used by the previous workers. Lunde and Kringstad ('39) concluded that the "filtrate factor" was distinct from the "anti-gray hair factor" since concentrates which promoted growth were not effective against hair depigmentation. Oleson, Elvehjem and Hart ('39) reported that pantothenic acid concentrates and other known vitamins were ineffective in preventing nutritional achromotrichia. Several

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investigators (Morgan and Simms, '40; Lunde and Kringstad, '40; Dimick and Lepp, '40; and Mohammad et al., '40) confirmed these results reporting that in addition to pantothenic acid, there is a second filtrate factor which is necessary for the maintenance of normal hair color in rats. The differentiation between these two filtrate factors was made before pure pantothenic acid was available and before the pantothenic acid requirement of rats was known.

György et al. ('40) were able to prevent and cure gray hair by feeding pantothenic acid concentrates and later György and Poling ('40 a) found synthetic calcium pantothenate to be active. Unna ('40), feeding synthetic calcium pantothenate, set the pantothenic acid requirement of the rat at 80 µg. per day and found that liver concentrates of known pantothenic acid potency were more effective in promoting growth than equivalent amounts of pure calcium pantothenate. Unna and Sampson ('40) were able to prevent and cure graying of the fur with 100 µg. of calcium pantothenate per day.

The complete maintenance of fur color has not been achieved by all investigators through the use of pure calcium pantothenate. Williams ('40) reported the failure of either pantothenic acid concentrate or synthetic calcium pantothenate to prevent or cure achromotrichia. Emerson and Evans ('41) have reported the prevention of pattern graying with calcium pantothenate but report that further supplementation is required to prevent "stippling." More complete prevention of graying was obtained by Dimick and Lepp ('40) when rice bran filtrate was used as a source of pantothenic acid than when pure calcium pantothenate was used. György and Poling ('40 b) were successful in curing the graying completely by feeding biotin when only a few scattered gray hairs remained after curing with pantothenic acid. Ansbacher ('41) was unable to prevent graying by supplying 500 µg. of calcium pantothenate per rat per day. Administration of p-aminobenzoic acid was effective against the symptoms he observed. Frost et al. ('41) reported a negligible effect of pantothenic acid alone against graying, but were able to get cures by

using liver extracts at levels which furnished as little as 40 μg. of pantothenic acid per day.

Somewhat over a year ago we attempted to perfect an assay procedure for the "anti-gray hair factor" which could be applied to naturally occurring materials. After many assays on animal tissues it was found that the activity of these samples paralleled very closely their pantothenic acid content as determined by the chick assay (Waisman et al., '39). When synthetic calcium pantothenate became available it was found to be effective against the graying we were observing. We wish to report in this paper our experiences with pantothenic acid in relation to both growth and graying in rats.

EXPERIMENTAL

The rats used for these studies were obtained from two sources, the black and piebald rats from our colony and the small number of albino rats used for growth studies from Sprague-Dawley. The rats were placed in individual screen bottom cages at 21 days of age and fed the ration ad libitum. The majority of the studies were conducted with basal ration 789 which consists of sucrose 73%, purified casein[2] 18%, salts IV[3] 4%, corn oil[4] 5%, thiamine 2 mg., pyridoxine 2 mg., riboflavin 3 mg., nicotinic acid 2.5 mg. and choline 1 gm. per kilogram. Two drops of halibut liver oil were given per week. The vitamins were added in solution to the casein and after drying the casein was ground with the sucrose and salts.

Synthetic calcium pantothenate[5] was fed daily in a supplement dish at the levels studied. All the rats were started on supplement at 21 days of age. On this basal ration the rats ceased to grow after 4 to 6 weeks and graying began to appear at about the same time. The gray hair sometimes appeared earlier in rats receiving low levels of calcium pantothenate than in those receiving the basal ration. However, spontaneous

[2] Labco from Borden and Company, New York City.

[3] Phillips and Hart (J. Biol. Chem., vol. 109, p. 657, 1935).

[4] Mazola.

[5] Merck's.

cures were frequently observed when these animals were continued on these low levels (20–40 μg.) for several weeks.

Table 1 summarizes the results obtained with 140 black and piebald rats and 68 albino rats on basal ration 789, plus graded levels of calcium pantothenate. The growth results include both the albino and piebald rats.

TABLE 1

The effect of various levels of calcium pantothenate on growth and hair color of rats fed ration 789.

LEVEL OF CALCIUM PANTOTHENATE	NO. OF RATS USED	PERCENTAGE WHICH GRAYED	AVERAGE WEEKLY GAIN IN GRAMS FOR 6 WEEKS
μg			
0	29	100	5.9
20	30	82	14.0
30	7	85	18.0
40	44	36	22.6
60	15	0	21.6
80	22	7	27.4
90	4	0	24.5
100	27	0	24.6
200	9	0	28.1
500	21	0	28.4

Rats receiving levels of calcium pantothenate above 40 μg. per rat per day showed no graying except in a few cases; at 40 μg. the results were variable with 36% graying, while at lower levels almost all rats turned gray. The requirement for growth agrees fairly well with the results of Unna ('40) although we obtained better growth on the optimum level. Some curative trials showed that definite signs of alleviation of graying occurred in from 3 to 6 weeks but usually about 4 weeks after the initiation of therapy (100–500 μg. per day). All rats were completely cured within a period of 8 weeks.

Studies with heated ration

The heated grain ration first introduced by Kline et al. ('32) is low in pantothenic acid. It has been used extensively in producing pantothenic acid deficiency in chicks, but has not been widely used for rats. Ansbacher ('40) reported

graying of rats on this ration when pyridoxine was added. We were able to cause graying of the fur of rats kept on the heated basal ration in 6 to 8 weeks. The ration consists of a basal mixture with the following percentage composition: ground yellow corn 58, wheat middlings 25 and crude casein 12 which were mixed and heated for 30 hours at 120°C., salts IV 4, and cod liver oil 1. To each kilogram of this mixture were added thiamine 2 mg., riboflavin 3 mg., pyridoxine 3 mg., nicotinic acid 25 mg., and choline 3 gm. Of twenty-three rats fed this basal ration only two did not become gray. Growth was slow, averaging 8.6 gm. per week for the first 6 weeks. Of the twenty-one rats receiving the basal ration to which had been added 10–20 mg. of calcium pantothenate per kilogram none showed signs of graying and the average growth for 6 weeks was 18.1 gm. per week.

Graying from other causes

Depigmentation of black and piebald rats has been reported (Gorter, '35; Keil and Nelson, '31; and Free, '40) on milk rations and attributed to copper deficiency. Free ('40) clearly differentiated between graying caused by a mineral deficiency and that caused by lack of an organic factor.

Graying was produced in piebald rats on whole milk supplemented with iron and manganese. The addition of copper at 50 μg. per day, as copper sulfate, completely prevented the condition but supplementation with 100 μg. of calcium pantothenate per day had no effect. The gray hair arising from lack of copper was clearly alleviated within 2 weeks after copper therapy was begun. The graying differed from that observed on basal ration 789 in that the hair had a definite brownish color instead of the silvery gray, observed in the vitamin deficiency.

Oettel ('36) reported graying of the fur of cats receiving hydroquinone. We attempted to repeat this observation using rats, but with no success. Rats receiving ration 789 plus adequate amounts of calcium pantothenate were fed 2–5 mg. of hydroquinone per day in aqueous solution. At the end of

5 weeks, growth was normal and there was no indication of graying. Raising the level of intake to 25–35 mg. of hydroquinone for 5 weeks produced no adverse effect on growth or hair color. Likewise on a milk ration no graying was noted when 10 mg. of hydroquinone was added to the mineralized milk each day. Martin and Ansbacher ('41) produced depigmentation in black mice by feeding a stock diet to which hydroquinone had been added. This was interpreted as a vitamin deficiency since p-aminobenzoic acid was effective in curing the gray hair. Although we used higher levels of hydroquinone our rats did not show the symptoms which the above workers noted in mice.

Following Ansbacher's ('41) report that p-aminobenzoic acid was effective against gray hair in rats which grayed while receiving as much as 500 μg. of calcium pantothenate per day, we fed this substance. It was not effective in relieving the gray hair noted in rats fed low levels (0–40 μg.) of pantothenic acid. When rats received adequate pantothenic acid a slight growth response was noted in rats from our colony. The average growth of four rats receiving ration 789 plus 80 μg. of calcium pantothenate was 19 gm. per week with a range of 16 to 22 while the average growth of rats receiving an additional supplement of 3 mg. of p-aminobenzoic acid was 27.8 gm. per week with seven of the eight showing a range of from 27 to 36 gm. per week. One rat grew at the rate of 17 gm. per week. Subsequent attempts to repeat this observation using rats from Sprague-Dawley have failed. With these animals ration 789 plus adequate pantothenic acid has given as high as 31 gm. per week growth, but added p-aminobenzoic acid has not increased growth. It should be pointed out that this might be due to the difference in strain or to previous nutritional history of the rats used. Thirty-one grams per week is better growth than we were able to obtain by feeding p-aminobenzoic acid to rats from our colony.

Balance studies

The large number of reports that natural crude concentrates were more effective in preventing gray hair than equiva-

lent amounts of pure pantothenic acid suggested the possibility of bacterial synthesis of pantothenic acid in the intestinal tract of the rats being studied. It seemed possible that these liver fractions were furnishing precursors or in some other manner stimulating synthesis of pantothenic acid which was being used by the animal. This, of course, would not explain the ineffectiveness of high levels of pure calcium pantothenate in preventing graying as some investigators had noted. It seemed unlikely that large amounts of pantothenic acid were available to the rats since animals on basal ration 789 often showed adrenal necrosis at death, a condition which is prevented by as little as 5 μg. of pantothenic acid per day (Mills et al., '40).

To test the possibility and to ascertain the requirement of the rat for pantothenic acid, balance studies were undertaken. The microbiological assay of Strong et al. ('41) was employed. Little quantitative data could be obtained on the pantothenic acid content of the feces, since added pantothenic acid was recovered to the extent of only 50 to 60%. However, the values as obtained indicated that considerable pantothenic acid was excreted in the feces. The amount was almost independent of the diet, since rats suffering from severe pantothenic acid deficiency excreted as much per gram of feces as rats receiving adequate pantothenic acid or liver fractions in which the pantothenic acid had been destroyed by autoclaving in acid solution.

The level of pantothenic acid in feces varied between 50 and 150 μg. per gram of air-dried feces as shown by this method or from 10 to 75 μg. per day. Corrected values would be approximately twice this high. The assumption that most of this pantothenic acid was synthesized by bacterial action in the cecum received support from the analysis of the contents of two portions of the intestinal tract. Three rats with severe pantothenic acid deficiency were used. The entire intestine from the stomach to the cecum contained from 1.5 to 5 μg. of calcium pantothenate, and the cecum and colon from 25 to 40 μg. It seems likely then that by far the greater part

of fecal pantothenic acid arises in the cecum and large intestine and may not be available to any appreciable extent to the rat.

Analysis of the urine was carried out simultaneously with fecal analysis. The urine was collected under toluene during 2–4 day periods and made up to a known volume by washing down the sides of the funnels. The microbiological assay was satisfactory, giving recoveries ranging from 89 to 116%[6] with an average value of 102%. Rats which had received basal ration 789 for several weeks excreted less than 1 µg. of calcium pantothenate per day, usually 0.3–0.4 µg. This amount was slightly less than that consumed in the ration, which contained about 0.2 µg. per gram. In no case was more pantothenic acid excreted in the urine than was consumed. This is another fact which makes utilization of pantothenic acid synthesized in the tract unlikely. Feeding various liver fractions did not increase urinary excretion above that noted when pure calcium pantothenate was fed at an equivalent level.

TABLE 2

Urinary excretion of calcium pantothenate in rats.

LEVEL OF INTAKE	NO. OF RATS	AVERAGE DAILY EXCRETION						AVERAGE
		1st wk.	2nd wk.	3rd wk.	4th wk.	5th wk.	6th wk.	
µg		*µg*	*µg*	*µg.*	*µg.*	*µg.*	*µg.*	*µg.*
20	6	.67	1.0	.6	1.4	2.2	1.7	1.3
40	6	2.1	2.3	1.9	2.6	4.6	8.5	3.7
60	4	1.6	3.6	6.9	9.8	8.7	8.2	6.4
80	2	4.4	7.7	24.3	8.5	4.4	7.3	9.4
150	2	10.2	91.0	52.0	65.0	50.0	68.0	54.8

In order to ascertain the requirement of the rat, the urinary excretion of pantothenic acid was followed in rats receiving varying levels of calcium pantothenate, administered both orally and intraperitoneally. A summary of the results is given in table 2.

Since no differences were observed between the values obtained when the calcium pantothenate was given orally and when injected, no differentiation is made in the table. It will

[6] The calculations were based on single determinations and these values show normal variation for such determinations.

be noted that there is a general increase in excretion from the first to the sixth week which indicates a decrease in requirement as the rate of growth decreases. The results indicate a marked increase in the urinary excretion of pantothenic acid as the level of intake is raised from 80 to 150 μg. Up to 80 μg. the excretion is very low, and at 150 μg. about 50 μg. are excreted indicating that 100 μg. is approximately the requirement for the growing rat. This is in good agreement with the growth experiments reported in a previous section.

DISCUSSION

It is evident from the data presented in the paper that rats on diets containing only the synthetic forms of the B vitamins require 80 to 100 μg. of calcium pantothenate per day for maximum growth. The actual requirement cannot be much higher than this value since a growth rate of 24 to 28 gm. per week is obtained. However, the requirement may be found to be lower in the presence of all essential factors.

An increase in the rate of growth of rats fed synthetic rations containing all the known vitamins has been obtained by adding liver extracts (Unna, '40; Elvehjem et al., '41). This growth increment may be small but it is always definite and indicates that these extracts contain additional factors required by the rat. However, successful reproduction has been reported by Jukes ('40). We have been able to raise rats through four generations with no pronounced difficulty on ration 789 together with 2 mg. calcium pantothenate per 100 gm. ration and α-tocopherol at 1 mg. per week. In all cases the rate of growth was not optimum but the young finally reached maturity and reproduced. Some of the mothers showed impaired lactation especially with the first litter.

In much of the earlier work the pantothenic acid requirement was assumed to be about 30 μg. per day. No increment in the rate of growth was obtained by raising the level from 30 μg. to higher levels. In our laboratory the lack of response was due largely to an inadequate supply of choline. The present studies, as well as those reported by Unna, show that

30 μg. may give fair growth but does not give the optimum rate or protection from graying. Levels of 40 μg. per day frequently caused graying to occur faster and to a more extensive degree than when no pantothenic acid was given. These facts may account for the earlier reports that pantothenic acid preparations were ineffective in the prevention of graying.

The fact that pantothenic acid alleviates hair depigmentation under the conditions of our experiments does not mean that this vitamin is the only organic factor concerned with the normal hair color. It is possible that different rations may give various deficiencies because of their effects on the intestinal flora. Studies on this problem are now in progress in this laboratory (Black et al., '41).

SUMMARY

Rats fed a synthetic ration deficient in pantothenic acid became gray in 4–6 weeks. Levels of calcium pantothenate above 40 μg. per day prevented or cured this condition. The rate of growth paralleled the level of pantothenic acid intake up to 80 μg. per day. Studies on the urinary excretion of this vitamin indicated that the daily requirement for the growing rat is approximately 100 μg. of calcium pantothenate per day.

Rats fed a heated grain ration showed graying which was likewise prevented and cured with synthetic calcium pantothenate. Copper deficiency caused a graying which did not respond to pantothenic acid but was rapidly cured by administration of copper.

Feeding hydroquinone failed to produce graying in rats receiving synthetic or milk rations. p-Aminobenzoic acid was entirely ineffective against the graying produced in rats by feeding a synthetic ration under our experimental condition.

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CRITERIA OF RESPONSE IN THE BIO-ASSAY OF VITAMIN E[1]

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Only during the past few years has any serious effort been made to improve the procedure for the bio-assay of vitamin E, based upon prevention of fetal resorption in the female rat, as established by Evans and Burr in 1927. Through proper regulation of the diet of breeding rats whose offspring are to be used for bio-assay purposes, it has been possible to dispense with the necessity of demonstrating sterility by means of a proven resorption and to use the first pregnancy period of the vitamin E deficient female for assay tests (Ringsted, '35; Bacharach et al., '37, '38; Mason and Bryan, '38, '40). This has greatly reduced the time and labor involved in the preparation of rats for bio-assay and for other experimental purposes. There still exists an unfortunate lack of uniformity regarding the duration of feeding of the test dose of vitamin E, and the most satisfactory means of evaluating the response obtained. The present discussion deals primarily with the latter problem.

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According to generally accepted criteria, a positive response implies the delivery of one or more progeny by a rat which otherwise would have undergone a resorption gestation. Uncertainty which may arise through cannibalistic proclivities of the mother can be avoided, in large part at least, by placing the parturient mother upon a raised coarse-screen floor such that the offspring fall out of reach of the mother soon after delivery. A negative response necessarily implies the intrauterine resorption of all implanted embryos. Methods for evaluating this "all or none" type of response as applied to bio-assay tests have been extensively discussed in recent papers by Bacharach and Allchorne ('38) and Bacharach ('38, '39).

Since the number of young delivered by rats showing a positive response will vary from one to many, and since negative responses must include uterine states varying from early resorption of fetal sites to those just falling short of a completed gestation, it is obvious that there exists a definite variation in the intensity of response of either type. No method has yet been devised for evaluating the latter. The efforts of Palmer ('37) to assess responses on the basis of data obtained at parturition, correlated with those obtained at autopsy immediately after delivery or after resorption, represent a step in this direction, even though the results offered no final solution to the assay problem. The studies presented in the present report represent an attempt (1) to establish more satisfactory criteria of response based upon data obtained at laparotomy or at autopsy on the sixteenth day of pregnancy, and (2) to determine whether such data can best be evaluated in terms of the "graded" or the "all or none" type of response.

EXPERIMENTAL

For use in the bio-assay tests reported in these studies, virgin E-deficient rats were standardized according to the following procedure. Rats of the breeding colony were reared and maintained on a diet of commercial dog biscuit known

to afford approximately four times the minimal daily requirement of vitamin E. Between the twelfth and fourteenth days of lactation the mother and offspring were transferred to the vitamin E deficient diet (casein, 20%; cornstarch, 48%; lard, 18%; salts, 4%; brewers' yeast, 8%; cod liver oil, 2%) upon which the progeny were subsequently maintained after weaning on the twenty-first day of lactation. The latter were mated with normal males when they had attained body weights of 150 gm. or better, usually within 30 to 50 days after weaning, and were used for assay tests during their first pregnancies. Evidence concerning the uniform and critical level of vitamin E depletion in such rats, and the factors involved in attaining this end, has been presented in earlier reports (Mason and Bryan, '38, '40; Mason, '40).

RESULTS

Gradations in positive and negative response. The results of a series of bio-assays designed to test the uniformity of response of our standardized virgin rats (table 1) confirm what has been stated above concerning the relationship which may exist between the size of the dose of vitamin E given

TABLE 1

Summary of bio-assay of a concentrate of vitamin E,[1] showing gradations in positive responses with increasing doses.

SINGLE DOSE IN MG.	NO. OF RATS USED	NO. RESORBED[2]	NO. DELIVERED	AVER. NO. OFFSPRING PER LITTER	NO. OF LITTERS WEANED[3]
3.75	5	5	—	—	—
5.0	10	8	2	2.5	—
6.25	10	6	4	3.3	—
7.5	10	2	8	3.4	2
10.0	11	1	10	6.6	3
15.0	11	—	11	6.9	8

[1] Supplied through the kindness of Dr. H. A. Mattill, Department of Biochemistry, State University of Iowa, Iowa City. Administered as a single dose to E-deficient rats on the first day of their first pregnancy.

[2] All resorptions were verified by autopsy.

[3] All litters showed vitamin E deficiency paralysis during the last few days of lactation.

and the intensity of the positive responses obtained, i.e., the number of young delivered at term. It is also worthy of note that the amount of vitamin E required for successful lactation was at least several times the minimal requirement for delivery at term, an observation which we have repeatedly confirmed (see also table 4).

During the course of another series of assay tests forty rats were subjected to laparotomy, and eighty others to autopsy, at different stages of pregnancy. A wide range of variation was noted in the gross appearance of the uteri in rats which, according to accepted criteria, would be regarded as exhibiting a negative response. Data obtained relative to size and state of the implantation sites, and weights of the uteri and their contents, suggested that the most satisfactory criteria for evaluating gradations in both negative and positive responses might be obtained by resorting to autopsy of all rats on the sixteenth day of pregnancy, at which time the body weights and uterine weights of untreated controls usually reached a maximum. On the basis of this experience the following procedure, briefly discussed in an earlier report (Mason, '39), was carried out in 600 routine bio-assay tests. At autopsy on the sixteenth day of pregnancy the cervix was severed at its mid-point and the full uterus, freed of mesentery and ovaries, was carefully weighed. After recording the live fetuses,[2] dead fetuses and resorptions the uterus was completely emptied and weighed. From these data two factors were obtained: (W) the weight of the uterine contents, and (N) the number of viable fetuses, when two or more were present. The presence of a single viable fetus, or of one or more dead fetuses, was disregarded except that their existence was reflected in the increased weight of the uterine contents. This practice was based upon the assumption that a single fetus surviving to the sixteenth day in a uterus in which resorption or recent fetal death had

[2] The combined weight of viable fetuses, together with intact membranes and placentae, varied from 0.92 to 1.34 gm. and averaged 1.145 gm. for 1222 fetuses weighed. The average weight of 160 fetuses of stock control rats at the sixteenth day was 1.248 gm.

occurred at other implantation sites had little likelihood of being delivered at term; this question is further discussed below. An arbitrary distinction was made between resorption sites and dead fetuses, depending upon whether the total weight of the fetal remnants and placenta was less, or greater, than 0.4 gm. Dead fetuses, even when approximating normal fetuses in size, were readily distinguished by their waxy white color and the pallor of the heart and liver, as well as by the absence of circulation in the blood vessels of the yolk sac, when examined with a dissecting microscope.

Attempts to evaluate gradations in response. After an extensive analysis of the data obtained, the formula, $\frac{W+N}{5} =$ uterine index, was adopted as the best means of numerically expressing the gradations in response observed. In the application of this formula to 600 routine bio-assay tests (table 2) it was found that responses were generally divisible into "negative", "subminimal" and "positive", based on uterine-index values less than 0.35, 0.35 to 0.99 and 1.0 or greater,

TABLE 2

Relationship between the number of live and dead fetuses and the distribution of uterine indices in 600 rats used in routine bio-assay tests and autopsied on the sixteenth day of pregnancy.

GROUP[1]	FETUSES PRESENT		DISTRIBUTION OF UTERINE INDICES					
			<.35 NEGATIVE		.35–.99 SUBMINIMAL		>.99 POSITIVE	
	Live	Dead	No. of rats	Range of indices	No. of rats	Range of indices	No. of rats	Range of indices
A	0	0	203	(.02–.34)	8	(.36–.46)	—	—
B	0	1	10	(.21–.34)	12	(.36–.60)	—	—
	0	2	—	—	12	(.38–.84)	—	—
	0	3 or >3	—	—	6	(.63–.97)	3	(1.06–1.51)
C	1	0	3	(.70–.32)	12	(.35–.61)	—	—
	1	1 or >1	—	—	18	(.40–.97)	1	(1.27)
D	2	0	—	—	4	(.87–.98)	6	(1.0 –1.25)
	2	1 or >1	—	—	—	—	17	(1.13–1.65)
	3 or >3	∓	—	—	—	—	285	(1.0 –5.65)

[1] The animals are grouped on the basis of degree of response, irrespective of dosage. Hence, no special significance is attached to the fact that the groups are arranged, in general, in order of increased dosage.

respectively. Untreated rats (negative controls), not included in the data of table 2, invariably gave values less than 0.35. It is evident that many responses which would be classed as negative on the basis of data obtained at the end of pregnancy can be recognized as subminimal by means of the uterine-index values or, in many instances, merely by gross inspection of the uterus at the sixteenth day of gestation. Likewise, the presence of dead fetuses in utero at this time, regardless of the absence or presence of viable fetuses, indicates a critical level of response. On the other hand, in the evaluation of graded responses, especially in rats receiving critical levels of dosage, the usefulness of the index values becomes impaired by the uncontrollable fluctuation of numerical values in the positive range primarily due to variations in the total number of implantation sites in different test animals (table 3). To a lesser extent, variations in the weight

TABLE 3

Showing the variation in total number of implantation sites (live fetuses, dead fetuses and resorption sites combined) observed in 1500 rats used in bio-assay tests and examined by laparotomy or autopsy at the sixteenth day of pregnancy.

TOTAL NUMBER OF IMPLANTATION SITES	NO. OF RATS	% OF RATS	TOTAL NUMBER OF IMPLANTATION SITES	NO. OF RATS	% OF RATS
1	14	0.93	8	239	15.93
2	18	1.2	9	351	23.4
3	19	1.27	10	319	21.27
4	23	1.53	11	193	12.87
5	36	2.4	12	66	4.4
6	55	3.67	13	35	2.33
7	120	8.0	14 or more	12	0.8

of the fetuses and annexa secondary to age differences of a few hours at this period of rapid growth may affect the values. In turn, these fluctuations overbalance the numerical gradations of subminimal responses. Numerous modifications of the uterine index formula designed to minimize the variables mentioned have offered no better solution to the problem of evaluating gradations in response.

In a series of bio-assay tests involving groups of eighteen to twenty rats at four different levels of E-dosage, it was found that the average of the uterine indices for each group when plotted against the dosage levels gave essentially the same curve as obtained by plotting in a similar manner the fertility rate; i.e., the per cent of rats in each group giving a positive response, as determined by the presence of two or more viable fetuses at the sixteenth day of pregnancy. As pointed out below, the latter criterion of positive response and that based upon parturition are virtually interchangeable. It thus appears that the uterine-index procedure offers no particular advantage over the customary method of treating the response as an "all or none" type and expressing the results in terms of fertility rate, as proposed by Bacharach and Allchorne ('38).

Criteria of response obtained at the sixteenth day of pregnancy. In evolving the uterine index discussed above, it was assumed that a single fetus surviving among three or more resorption sites[3] at the sixteenth day had little likelihood of being delivered at term; whereas at least one of two or more viable fetuses would undoubtedly be delivered. This postulate was based upon a preliminary series of sixty-two bio-assay tests on paired littermates, one of each pair being autopsied at the sixteenth day and the other allowed to continue to term. When positive responses were based upon the presence of at least two living fetuses at the sixteenth day and upon the delivery of at least one at term, respectively, the proportion of negative and positive responses was essentially the same (table 4).

In order to obtain more direct evidence on this point, 215 rats used in routine bio-assay tests were laparotomized on the sixteenth day of pregnancy. Complete resorption was

[3] In concurrence with a suggestion made to the writer by Dr. L. S. Palmer, it seems advisable to exclude from the computation of bio-assay results all rats which possess less than four implantation sites, regardless of whether the latter are represented by resorptions or fetuses. The number of animals eliminated from tests by adhering to this rule is not great, amounting to but 3.4% in the 1500 rats represented in the data of table 3.

TABLE 4

Paired bio-assay tests of a concentrate of vitamin E, comparing criteria of positive response based upon (1) delivery of one or more offspring at the end of pregnancy, and (2) the presence of two or more viable fetuses at the sixteenth day of pregnancy.

DOSE IN MG.[1]	RATS CONTINUED TO TERM		RATS AUTOPSIED ON SIXTEENTH DAY OF PREGNANCY		
	No.	Result	No.	Result	Implantation sites expressed as Living-Dead-Resorptions[2]
10	3	3 neg.	3	3 neg.	0-0-10; 0-0-10; 0-0-9
15	4	4 neg.	4	4 neg.	0-0-6; 0-0-11; 0-0-13; 1-1-3
20	9	7 neg. 2 pos.[3]	9	5 neg. 4 pos.	0-0-10; 0-0-9; 0-1-6; 1-1-7; 1-5-4 4-1-6; 7-2-1; 7-0-2; 8-0-2
30	9	2 neg. 7 pos.[4]	9	2 neg. 7 pos.	1-0-8; 1-3-5 2-1-14; 4-0-6; 4-0-6; 7-0-0; 7-2-0; 8-0-2; 9-0-0
40	6	6 pos.[5]	6	6 pos.	2-2-6; 5-0-4; 7-0-1; 8-0-2; 8-1-2; 9-0-1

[1] Fed as a single dose on the fourth day after positive mating.

[2] This method of recording the response conveys a convenient picture of the uterine state at autopsy. It should be stated, however, that occasional resorption sites are of normal occurrence in rats receiving an adequacy of vitamin E. Of seventy-five normal stock rats of the breeding colony autopsied on the sixteenth day of pregnancy, forty-two possessed one or more sites of advanced resorption interspersed between normal fetal sites.

[3] Only one offspring in each litter. Both died within 2 days.

[4] Average of 5.7 young per litter, none of which survived more than 4 days.

[5] Average of seven young per litter. Five litters successfully weaned, but paralysis of young appeared in all instances.

found in 103, and one or more viable fetuses [4] encountered in the remaining 112 which were continued to term and placed upon an elevated screen to permit the newborn to fall out of reach of the mother as delivered. Of seventy-two rats possess-

[4] It was sometimes difficult to determine at laparotomy whether or not certain fetuses were viable, even though both uterine horns were routinely withdrawn through the incision and carefully examined. Unless the operator was convinced that death had occurred, the fetuses were classed as living.

It is worthy of note that none of these rats, and none of several hundred other rats coming to delivery after receiving critically low dosage of vitamin E at various intervals prior to or during pregnancy, has exhibited the prolongation of gestation described by Barrie ('38) in rats under similar conditions and ascribed by her to impaired function of the corpus luteum secondary to anterior pituitary deficiency. Parturition invariably occurred between the twenty-first and twenty-third day, irrespective of the viability of the fetuses in utero. Since this is also the experience of many other investigators, the gestational abnormalities observed by Barrie can best be attributed to some experimental factors other than an inadequacy of vitamin E.

ing more than four viable fetuses at laparotomy, fifty delivered an equivalent number at term; while twenty-two delivered fewer (usually one or two) than were present at operation. Of the remaining forty rats, with one to four live fetuses at laparotomy (table 5), twenty would also be positive by either

TABLE 5

Record of forty rats used in E-assay tests and possessing less than five viable fetuses on the sixteenth day of pregnancy, showing the distribution of live fetuses-dead fetuses-resorption sites at laparotomy and the number of offspring delivered at term (indicated in parentheses).

RAT NO.	UTERUS	RAT NO.	UTERUS	RAT NO.	UTERUS	RAT NO.	UTERUS
2158	4-0-1 (4)	2668	2-1-2 (2)	2735	2-1-6 (0)	2862	1-0-5 (0)
2702	4-0-2 (4)	2813	2 1-4 (2)	2983	2-2-0 (0)	3032	1-0-7 (0)
2739	4-0-7 (4)	2822	2-1-5 (2)	2686	2-2-8 (0)	2670	1-1-9 (0)
3004	4-2-0 (4)	2715	2-0-2 (2)	2754	2-3-3 (0)	2741	1-2-4 (0)
2595	4-2-1 (3)	2740	2-0-2 (1)	2964	2-3-3 (0)	2838	1-2-5 (0)
2847	4-0-2 (3)	3001	2-0-5 (1)	2687	1-0-4 (1)	3049	1-2-4 (0)
2605	4-1-4 (2)	2988	2-0-7 (1)	2714	1-0-8 (1)	2743	1-3-5 (0)
2616	4-1-5 (2)	2691	2-1-7 (1)	3050	1-1-2 (1)	2872	1-3-5 (0)
3042	3-2-2 (3)	2969	2-2-4 (1)	2581	1-0-0 (0)	2894	1-3-4 (0)
2562	3-2-4 (2)	2700	2-5-3 (1)	2709	1-0-1 (0)	2717	1-4-6 (0)

criteria and twelve others negative, leaving eight animals whose responses differ when measured by the two criteria. These comprise five (out of fifteen) which failed to deliver either of two viable fetuses, and three (out of fifteen) which delivered a solitary fetus, present at the time of operation. Since these eight animals represent but 7% of the 112 with viable fetuses at laparotomy, and only 3.5% of the 215 rats operated upon after receiving vitamin E dosage close to the threshold level, it may be assumed that the two criteria are virtually equivalent in evaluating the "all or none" type of response in routine bio-assay tests.

Advantages of the 16 day autopsy procedure. On the other hand, the state of the uterus at the sixteenth day of pregnancy more clearly reveals the nature of individual responses, especially those of the subminimal type, than does the information obtained at the end of pregnancy. This shortened assay procedure possesses other advantages which warrant brief comment: first, a reduction of about 6 days in the time required

for completion of assay tests (of particular value in preliminary or exploratory types of assays) with the resultant saving in diet and animal care, together with the economy in cage space, since assayed rats can be grouped instead of being caged individually for data on delivery; second, the opportunity to observe the actual state and number of implantation sites, permitting the elimination of rats with less than four implants and the recognition of sub-minimal types of response. Although there seems to be no alternative but to consider the latter as negative in the final evaluation of assay results, they afford valuable and trustworthy indicators of a critical level of dosage. The same may be said of positive responses associated with the presence of dead fetuses at the sixteenth day.[5] A third advantage lies in the prompt detection of states of pseudopregnancy with bleeding[6] which, unless vaginal smears are followed daily, can easily be misinterpreted as resorption pregnancies and incorrectly recorded as negative responses unless the uterus is inspected during gestation or soon afterward. The usefulness of the abbreviated bio-assay procedure proposed is exemplified by a series of studies presented elsewhere (Mason, '42).

[5] A separate report will deal more specifically with a hitherto undescribed hemorrhagic state which I have frequently observed in both dead and living fetuses of low-E mothers. Since the incidence of this hemorrhagic state increases as the dosage of vitamin E is decreased, and since it cannot be prevented by administering relatively large doses of vitamin C or vitamin K to the mother, there is good reason to believe that it is a pathognomic sign of vitamin E deficiency and the cause of fetal death during late pregnancy. Its occurrence in association with positive assay responses constitutes a helpful indicator of critically low levels of dosage.

[6] Although various investigators have noted the frequent occurrence (10%–15%) of pseudopregnancy in rats mated for vitamin E assays, no particular reference has been made to the vaginal bleeding sometimes associated with this state. This latter phenomenon is characterized by the appearance of blood in the vaginal smear 1 or 2 days prior to the end of the 10- to 17-day period of pseudopregnancy. Autopsy at the onset of bleeding consistently reveals one or more small deciduomata which, in the course of their regression, give rise to the blood observed. This undoubtedly corresponds to the condition described by Evans ('28) as occurring in 52% of E deficient rats, and in but 3% of normal rats, made pseudopregnant by mating with vasectomized males. The frequency of bleeding in my pseudopregnant E deficient rats has been much less (about 5%) than observed by Evans.

SUMMARY AND CONCLUSIONS

1. Attempts to evaluate recognized gradations in negative and positive responses to vitamin E administration, based upon the number of live fetuses and the weight of the uterine contents at the sixteenth day of pregnancy in 600 rats used in assay tests, gave results not much superior to those obtained by treating responses as an "all or none" type, except for the recognition of a sub-minimal type of response otherwise classed as negative.

2. On the other hand, the presence of two or more viable fetuses in utero at the sixteenth day of pregnancy was found to afford a criterion of positive response essentially equivalent to, and in certain instances more equitable than, that based upon delivery of progeny at term.

3. In addition to advantages arising from the shortening of the test period for bio-assay, with the resultant economy of diet and animal care, examination of the uterus at the sixteenth day gives information especially pertinent to critical levels of response and prevents misinterpretation of certain atypical uterine states as negative responses to dose.

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DISTRIBUTION OF VITAMIN E IN THE TISSUES OF THE RAT[1]

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Knowledge concerning the distribution and storage of vitamin E in the animal body is decidedly limited. The rat bio-assay test is laborious and chemical methods have failed to give consistent results when applied to the detection of the vitamin in tissues. The pioneer studies of Evans and Burr ('27), in which the rat bio-assay test was applied to tissues from rats, cattle, pigs and sheep, indicated that vitamin E is widely distributed in the animal body but is never present in very great concentration in any one organ or tissue. Musculature, body fat and liver, in the order named, contained appreciably more vitamin than other tissues. In like manner, vitamin E has been demonstrated in the milk, musculature and adipose tissue of goats fed a normal diet, but not in the tissues of goats reared on a diet deficient in the vitamin (Underbjerg, Thomas and Cannon, '39). The data presented in these reports cannot be treated quantitatively, due to diversity in the vitamin E intake of the donor animals and to variations in the time of feeding of the test animals.

The various chemical tests for vitamin E have not given harmonious results when applied to the quantitative estimation of the vitamin in vegetable oils and concentrates of the latter (Smith and Bailey, '39). Uncertainty as to the speci-

[1] This investigation was aided by a grant to Vanderbilt University School of Medicine from the Division of Medical Sciences of the Rockefeller Foundation.

ficity of the reactions in the presence of many interfering substances has greatly restricted their application to animal tissue. The fact that such methods permit no discrimination between α, β, and γ tocopherols, which differ somewhat in their biological activity, prevents close correlation between chemical and biological evaluations of vitamin E. The spectroscopic method has demonstrated tocopherols in the body fat, muscles, urine, feces and blood of rats previously fed sources of the vitamin, but has revealed none in the liver or other tissues, suggesting that the tocopherol molecule is readily broken down or ineffectively stored by the organism (Cuthbertson, Ridgeway and Drummond, '40; Moore and Rajagopal, '40). Furthermore, results obtained by spectroscopic and colorimetric methods are not always in close accord. On the other hand, Karrer, Jaeger and Keller ('40) reported the presence of considerable tocopherol in the liver and musculature of horses and cattle as demonstrated by colorimetric and by potentiometric methods, the two procedures giving comparable results.

In view of the inadequacies of chemical means for evaluating vitamin E, any reinvestigation of its distribution in the animal body entails the use of the longer bio-assay method. Certain modifications of the latter have increased its reliability and permitted a significant shortening of the assay period (Mason, '42). These improvements have greatly facilitated the studies presented in this report, which are concerned with the biological estimation of vitamin E in organs and tissues of rats maintained on three different levels of vitamin E intake. The rat was selected as a basis for study since its need for vitamin E is indisputable and its minimal daily requirements reasonably well established for both sexes (Mason, '40). It was hoped that the results obtained from such a study might supplement existing knowledge concerning the metabolic utilization of the vitamin and, at the same time, afford a standard for testing the reliability of chemical methods for detecting its presence in organs and tissues.

EXPERIMENTAL

The three groups of rats from which tissues were obtained for bio-assay tests will be referred to as: (1) "low-E" rats, reared on a commercial dog food;[2] (2) "high-E" rats, fed for a period of 2 to 4 months on a diet of fresh, raw wheat germ supplemented with a small amount of casein and inorganic salts; and (3) "excess-E" rats, reared to 3–4 months of age on a diet containing 5% of mixed tocopherols.[3] It was estimated that the daily intake of vitamin E for the three groups was approximately 4, 100 and 10,000 times the minimal daily requirement, respectively.

Organs and tissues obtained from the donor rats were trimmed, weighed, placed in glass vials, frozen and stored in a refrigerator at 0° C., except when removed long enough to obtain portions for feeding. The tissues were usually fed within 2 weeks following their removal from animals, although there was no indication that longer periods of storage resulted in any loss in vitamin E content. Body fat was mixed with a casein-cornstarch-yeast mixture in order to be effectively administered. Blood was fed in the same manner, or as a concentrated alcohol-ether extract.

Standardized E-deficient females, prepared as described elsewhere (Mason, '42), were mated when weighing from 150 to 175 gm. and fed the tissues to be assayed during the first to tenth days of their first pregnancy. Aliquot parts of the total dose, the number varying with the size of the latter, were fed as daily doses about equally distributed around the fifth day of pregnancy.[4] The E-deficient diet was removed 3 to 6 hours before the test substance was fed, and a similar

[2] "Wayne Blox", manufactured by Allied Mills, Inc., Chicago.

[3] I am indebted to Dr. P. L. Harris of Distillation Products Inc., Rochester, N. Y., for his kindness in providing these rats. Due to the limited number of animals available, assays were restricted largely to liver and muscle.

[4] This procedure was followed on the basis of evidence (unpublished studies) indicating that single doses of vitamin E become increasingly more effective the later they are administered during the first 8 days of pregnancy. Throughout these studies the day following that on which sperm were found in the vaginal smear, or a vaginal plug observed, is referred to as the first day of pregnancy.

AMT.	DA. BED	UTERUS

Liver (low E)

140	1–10	5–1– 1
120	3–10	5–0– 3
120	3– 9	8–0– 1
120	3– 9	0–0–10
120	2– 8	0–0– 8
110	2– 8	9–0– 3
110	3– 9	0–0– 9
110	3– 9	0–0–10
100	3– 8	4–3– 2
100	3– 8	0–0–10
100	3– 8	0–0–11
100	3– 8	0–0–11
80	3– 7	0–0– 8
80	3– 7	0–0– 9
60	2– 7	0–0–10
50	3– 7	0–0–10
50	3– 7	0–0– 9
40	4– 6	0–0– 9
30	5– 6	0–0–15
20	4– 6	0–0– 7

Liver (high E)

40	1–10	7–0– 0
30	1– 5	9–0– 0
30	4– 6	9–0– 1
20	3– 5	9–0– 0
15	5	10–0– 0
10	5	9–0– 3
10	5– 6	8–0– 1
10	5– 6	8–0– 0
10	5– 6	9–1– 0
10	5– 6	0–0– 9
10	4– 6	0–0– 8
7.5	5	9–0– 0
7.5	5– 6	8–1– 1
7.5	5– 6	0–1– 5
7.5	5– 6	0–0– 9
7.5	5– 6	0–0– 9
7.5	7– 8	0–0– 9
7.5	5– 6	0–0– 9
7	5	0–0– 7
6	5	0–0–12
5	5	2–1– 2
5	5– 6	1–3– 5
5	5– 6	0–0– 8
5	5	0–0–10
5	5	0–0–11
3	5– 6	0–1– 5
3	5– 6	0–0– 7
3	5	0–0–10

Muscle (low E)

100	1– 9	2–1– 4
80	1– 8	2–1– 5
70	3– 9	5–1– 1
70	3– 7	8–0– 1
70	3– 7	0–0– 2
70	3– 7	0–0– 5
60	3– 7	7–1– 2
60	3– 6	4–1– 5
60	4– 8	2–3– 2
60	4– 8	5–4– 0
60	4– 8	7–0– 1
60	4– 8	0–0– 6
60	3– 5	0–0–10
60	4– 8	1–0– 7
50	4– 6	0–1– 8
50	4– 7	0–0– 8
50	4– 7	0–0– 8
40	4– 7	0–0– 8
40	3– 6	0–0–10

Muscle (high E)

60	1– 9	6–0– 2
60	1– 7	10–0– 0
40	5– 7	4–0– 1
40	4– 6	9–0– 0
30	4– 7	7–0– 0
30	4– 6	8–0– 1
20	4– 6	8–0– 0
20	6– 7	10–0– 0
20	4– 6	7–0– 4
20	4– 5	0–0–10
15	4– 5	7–0– 1
15	6– 7	0–2– 8
15	6– 7	0–0–11
15	6– 8	2–2– 0
15	5– 7	2–0– 7
10	7	0–1– 7
10	6	0–0– 6
10	4	0–0– 1
7	6	0–0– 7

Kidney (low E)

80	3– 9	9–3– 1
60	2– 8	3–1– 6
60	4– 8	4–2– 2
60	4– 8	0–0–10
60	3– 7	0–0–10
50	4– 8	0–0– 9
45	4– 7	3–5– 1
40	4– 7	0–0– 9
40	4– 6	0–0– 9
30	5– 8	0–0– 9

Kidney (high E)

20	3– 5	9–1– 0
20	4– 6	3–0–10
20	4– 6	0–0–12
15	5– 7	10–1– 1
15	4– 6	7–2– 1
15	4– 6	5–2– 2
15	4– 6	3–0– 7
15	3– 5	0–0– 9
10	5– 7	6–0– 3
10	5– 6	0–0– 9
10	5– 6	0–0–10
10	6– 8	0–0– 8
10	4– 6	0–0–10

Body fat (low E)

50	6–13	10–0– 1
45	1–10	1–1– 8
40	1–10	0–1– 8
30	2–12	0–0–11
30	1–10	0–0–11
20	1–10	0–0– 6
20	0– 7	0–0– 9

Body fat (high E)

35	1– 9	9–0– 0
30	1–10	6–0– 4
20	2– 8	10–0– 0
20	1–10	10–0– 0
20	1–10	6–0– 0
20	3–10	6–0– 2
15	3– 8	7–1– 3
15	3– 8	0–1– 7
15	3–10	0–0– 9
15	5–10	0–0– 8
15	5–10	0–0– 9
15	1– 7	0–0–10
10	5–10	0–0– 6
10	5– 9	0–0–10
10	5–10	0–0– 8
10	4– 5	0–0– 9

Pancr. and Thym. (low E)

40	4– 7	6–0– 4
40	4– 7	0–0– 8
30	4– 7	0–0– 9
30	3– 7	0–0– 8

Pancr. and Thym. (high E)

30	4– 6	7–0– 1
13	5– 7	9–0– 0

Heart (low E)

40	4– 7	8–0– 2
40	5–10	7–0– 2
30	5– 7	9–0– 1
30	4– 6	0–0– 9
30	4– 7	0–0– 4
27.5	4– 7	0–0– 7
10	4	0–0–11

Heart (high E)

15	4– 6	5–0– 1
10	5– 7	8–0– 1
10	4– 6	2–5– 3
10	7	2–0– 2
10	5– 7	0–0–11
8	5– 6	0–0–12
7.5	5– 6	0–3– 6

Lung (low E)

45	4– 8	7–1– 1
40	4– 7	8–0– 0
40	4– 7	7–0– 0
40	3– 6	6–3– 2
35	4– 7	0–1– 7
35	4– 7	0–0– 7
30	4– 7	1–2– 4
30	5– 7	1–1– 2
30	4– 6	0–1– 9
30	4– 7	0–0– 9
30	4– 7	0–0– 8

Lung (high E)

15	7	11–0– 0
15	4– 6	9–0– 0
15	4– 6	8–0– 0
10	4– 6	11–0– 0
10	6– 7	6–1– 1
10	5– 6	4–0– 2
7.5	5– 7	7–0– 2
7.5	5– 6	5–0– 3
7.5	6– 7	0–0–12
7.5	5– 6	0–0–10
7.5	5– 6	0–0– 8
5	5– 6	0–0–13

Spleen (low E)

35	5– 8	8–0– 0
35	3– 7	2–2– 4
35	4– 7	1–2– 5
32	5– 8	8–1– 3
30	5– 8	8–0– 0
30	4– 7	7–2– 3
30	4– 6	2–0– 5
30	3– 7	0–0– 8

Spleen (high E)

25	4– 9	2–0– 0
17.5	4– 6	9–0– 0
14	5– 7	9–0– 0
7.5	6– 7	0–0– 8

TABLE 1 (*continued*)[1]

AMT.	DA. FED	UTERUS
Testis (low E)		
60	4–8	7–1–2
60	4–8	2–0–6
60	4–8	0–1–10
60	3–8	0–2–7
60	4–8	0–3–6
45	4–8	0–0–10
35	4–6	0–0–7
30	4–6	0–0–9
30	5–7	0–0–4
20	5–6	0–0–8
Testis (high E)		
23	5–6	8–0–0
20	4–6	0–1–2
15	4–6	6–0–2
15	4–6	1–3–5
15	3–4	0–0–9
15	4–6	0–0–12
15	4–6	0–0–11
15	4–6	0–0–10
12	4–6	0–0–11
9	5–6	0–0–9
Epididymis (low E)		
54	5–8	7–1–1
40	4–8	0–0–10
40	4–8	0–0–8
30	3–8	0–0–4
25	4–7	0–0–8
Epididymis (high E)		
15	4–7	4–2–1
15	4–6	0–0–11
11.5	4–6	2–2–4
10	4	1–0–8
8	5–6	0–0–6
Prost. and S.Ves. (low E)		
60	4–8	7–0–1
50	4–8	0–1–6
44	3–6	0–0–10
Prost. and S.Ves. (high E)		
35	4–8	6–0–3
27	5–7	11–0–0
20	4–6	0–0–9
16	4–6	0–0–12
Placenta (high E)		
20	5–7	7–0–0
10	5–6	2–3–3
10	5–6	0–0–10
10	5–6	0–0–9
6.5	5–6	0–0–6
5	5–6	0–0–8

AMT.	DA. FED	UTERUS
Uterus (high E)		
10	5–6	8–0–2
7	5–6	0–0–7
6	5–6	4–0–2
5	5–6	0–0–7
5	5–6	0–2–6
Mammary gl. (low E)		
35	4–7	4–2–3
35	4–6	0–0–9
30	4–6	0–0–9
20	4–6	0–0–10
10	4–6	0–0–10
Mammary gl. (high E)		
10	5–6	11–0–0
10	5–7	8–0–0
7.7	5–6	8–0–0
5	5–6	12–0–0
5	5–6	7–0–1
5	5–6	4–2–0
5	8	2–3–3
5	6–7	0–1–2
3.5	5–6	9–0–0
3.5	5–6	1–2–4
3.5	4–6	0–0–9
2	5–6	0–0–11
2	5–6	0–0–4
Suckling young (low E)[2] *24–48 hrs.*		
80	3–8	10–0–1
80	4–8	8–0–1
80	2–7	6–0–0
60	4–7	8–0–2
60	4–8	8–0–0
60	4–6	7–0–2
60	4–6	7–0–1
45	4–7	6–1–2
45	4–7	0–0–12
45	5–7	0–0–10
45	4–6	0–0–9
45	4–6	0–0–8
30	5–6	0–0–11
30	5–6	0–0–9
30	5–6	0–1–8
Brain and Sp. Cord (high E)		
46	3–7	5–0–5
40	3–6	0–0–10
22	4–7	0–0–7
12	1–8	0–0–9
2.2	6	0–0–10

AMT.	DA. FED	UTERUS
Blood (high E)		
80[3]	1–10	0–0–2
60[3]	1–10	0–0–9
60[3]	1–10	0–0–2
40[3]	1–10	0–0–11
40[3]	5–7	0–0–10
40[3]	3–5	0–0–10
40[3]	1–10	0–0–9
36	1–9	7–1–0
30	2–9	0–0–12
30	5–7	0–0–10
20	2–7	0–0–9
13	5	0–0–11
MASSIVE DOSE OF E[4]		
Liver		
5.5	5–6	9–0–1
4.5	5–6	3–0–4
4	5–6	11–0–1
2	5	12–0–0
2	5–6	10–0–0
2	5–6	7–0–0
1	5	11–0–0
1	5	9–0–1
1	5	8–0–2
1	5	6–0–2
1	5	3–1–0
0.6	5	0–1–10
Muscle		
10	5–6	10–0–2
10	5–6	9–0–1
10	5–6	1–1–8
5	5–6	9–2–0
5	5–6	8–0–0
5	5–6	0–0–11
5	5–6	0–0–11
5	5–6	0–0–9
2.5	5–6	0–0–8
Body fat		
7	4–7	8–0–0
Testis and Epid.		
8	5–6	0–0–7
8	5–6	0–0–4
Brain		
9.8	5–6	0–0–8
Blood		
19	5–9	6–0–3
4	5–6	0–0–9

[1] The figures in each line indicate, respectively, the number of grams of fresh tissue fed, the days of pregnancy over which the total dose was divided, and the number of viable fetuses, dead fetuses, and resorption sites observed at the sixteenth day of pregnancy.

[2] Data for twenty-six assays on newborn of low-E and high-E rats, and 24-hour nurslings of high-E rats, have been presented elsewhere (Mason and Bryan, '40, table 2).

[3] These tests were made on an ether-alcohol extract of whole blood, concentrated in vacuo, because of difficulties in feeding large quantities of whole blood. It now seems probable that exposure to light during the process of concentration destroyed much of the vitamin present.

[4] Tissues of "excess-E" rats. The limited supply of animals prohibited more extensive analysis of the tissues. It is estimated that the mean fertility dose of liver and of muscle

period elapsed between consumption of the latter and restoration of the experimental diet. The animals were observed at frequent intervals in order to replace any assay material lost through the screen floor of the cage, and to assure complete consumption of the daily dose.

Vaginal smears were obtained on the fourth, fifth, and tenth to thirteenth days after positive mating. The rats were autopsied or laparotomized at the sixteenth day of pregnancy, and the presence of two or more viable fetuses taken as a criterion of positive response. The latter has been shown to be equivalent to, but much more informative than, the delivery of one or more offspring at term (Mason, '42).

BIO-ASSAY RESULTS

Because of inadequacy of information on the duration of feeding and the individual responses in bio-assays of animal tissues previously reported in the literature, the data obtained have been presented as fully as possible (table 1). Assay tests on certain organs and tissues were unfortunately limited by their small size and by difficulties either in obtaining sufficient amounts for assay purposes or in securing a sufficiently large intake of fresh tissue (e.g., fat) during the first 10 days of pregnancy. The appearance of pseudopregnancy frequently frustrated attempts at assay of valuable material. However, an attempt has been made to estimate the "mean fertility dose" of each tissue tested; that is, the amount of tissue which might be expected to produce a positive response in 50% of a reasonable number of rats tested (as defined by Bacharach, '38). Table 2 presents a summary of these estimates, based upon the 312 assay tests of table 1 supplemented by twenty-six others previously reported (Mason and Bryan, '40). When due allowance is made for animal variations in response to critical levels of dosage and for the small gradations in dosage levels used, in comparison with those generally employed in bio-assay tests, many of these estimates seem equitable. Certain others, indicated in table 2 by a question mark, must be regarded as conjectures. Since the

method of preparation of virgin rats for assay tests unequivocally excludes the possible occurrence of "first litter fertility" (Mason and Bryan, '38, '40; Mason, '42), the responses recorded in table 1 can be accepted with confidence.

TABLE 2

The estimated "mean fertility dose" of tissues from low-E and high-E rats, expressed as grams of fresh tissue, and the calculated difference in concentration of the vitamin.

TISSUE	LOW-E RATS GM. OF TISSUE	HIGH-E RATS GM. OF TISSUE	DIFFERENCES IN E-CONTENT (TIMES)
Liver	120	8.5	14.1
Muscle	60	15	4.0
Kidney	60	13	4.6
Body fat	50	17	2.9
Pancreas and thymus	45 ?	10 ?	4.5
Heart	35	9	3.9
Lung	35	8	4.4
Spleen	30	10 ?	3.0 ?
Testis	60	20 ?	3.0 ?
Epididymis	55 ?	15 ?	3.7 ?
Prostate and sem. ves.	60 ?	20 ?	3.0 ?
Placenta	—	13 ?	—
Uterus	—	8 ?	—
Mammary gland	35 ?	4	8.7 ?
Newborn	200 [1]	50 [1]	4.0
Suckling young (24–48 hours)	50	15 [1]	3.3
Brain and spinal cord	—	50 ?	—
Blood (whole)	—	40 ?	—

[1] Based upon assay data presented in a previous report (Mason and Bryan, '40; table 2).

Several interesting facts are apparent from these data. (1) Substances possessing the biological activity of vitamin E are widely distributed throughout the tissues and organs of the rat; the concentration of the vitamin in those tissues which undergo pathological alteration after E-deprivation (e.g., fetus, testis, musculature, uterus, brain and spinal cord) differs in no significant manner from that in other tissues. This stands in striking contrast to what is known concerning the distribution of vitamin A in the rat (Popper and Greenberg, '41).

(2) At low levels of intake the liver contains about one-half as much vitamin E per gram of fresh tissue as the skeletal muscles, kidney, body fat, testis, epididymis, prostate and seminal vesicle, and about one-fourth as much as the heart, lung and spleen. Apparently, the metabolic needs of body tissues of low-E rats are satisfied at the expense of liver storage, suggesting that four times the minimal daily requirement is considerably below the optimal requirement. This would be in full accord with what is known concerning other vitamins.

(3) Increasing the daily intake from four to 100 times the minimal requirement permits a fourteenfold increment in liver storage but raises that in the other tissues only three to four and one-half times. At an intake approximately 10,000 times the minimal (see table 1), the storage in liver and muscle increases to 150 and to twelve times, respectively, that in low-E rats.

(4) In both low-E and high-E rats the active mammary gland possesses a greater storage capacity for E than the liver. In view of observations that mammary transfer is decidedly limited (Mason and Bryan, '40), it would appear that concentration of the vitamin in the glandular tissue greatly exceeds that in the secretion produced.

(5) The E-content of newborn rats is much less than that of the adult tissues examined[5] and is augmented to the same degree as the latter (exclusive of the liver and mammary gland) by increased intake of the vitamin. A fourfold increment occurs in offspring of both low-E and high-E rats after 24–48 hours lactation, bringing the E-content up to that of adult muscle, body fat and kidney. It cannot be stated, however, what proportion of the vitamin existed in the stomach contents and what proportion was actually stored in the tissues of the young rat.

[5] The few data available suggest that the vitamin E content of nervous tissue and of whole blood more closely resembles that of the newborn than that of other adult tissues.

DISCUSSION

These studies confirm the conclusions of Evans and Burr relative to the wide distribution of vitamin E in the body of the rat but indicate that musculature and body fat, which they considered to possess the greatest concentration of the vitamin, actually contain an amount similar to or less than that present in other tissues and organs, whether the previous intake be low or high. Their low values for the vitamin in rat liver, which they recognized as being incompatible with the remarkable storage capacity of this organ for other fat soluble vitamins, may be explained by the fact that their donor rats received a sub-optimal intake of vitamin E not unlike that of the low-E rats used in the present study. Although the liver constitutes the chief repository for vitamin E when the intake is optimal or greater, its storage capacity seems to be decidedly less than that for vitamins A and D. It is obvious, however, that its degree of saturation represents the most reliable index of previous intake and storage. Bio-assays of human and monkey livers by Mr. R. Terry in this laboratory (unpublished studies) have demonstrated a storage about intermediate between that observed in low-E and high-E rats. On the other hand, livers from dogs with bile fistulae have invariably given negative responses at similar and higher levels of feeding, which confirms earlier observations of Greaves and Schmidt ('37) concerning the failure of bile-fistula rats to absorb vitamin E.

It cannot yet be stated what significance may be attached to the disparity between the concentration of the vitamin in newborn rats and that in the uterus and placenta, or between that in nurslings and in the mammary gland. No adequate explanation can be offered for the exceptionally high content of E in the latter tissue.

The small proportion of ingested vitamin which is stored by the rat, and the fate of the remainder, constitute problems warranting further investigation. Assuming that the various tissues tested in the present studies would constitute one-half

the weight of the rat, and assuming that the remaining tissues possessed an equivalent amount of the vitamin, it is estimated that the total storage in low-E and high-E rats of 300 gm. body weight would amount to about three and fourteen mean fertility doses of vitamin E, respectively. In the case of the high-E rats, this would represent only about three times the daily intake. In confirmation of the conclusions of Moore and Rajagopal ('40) and of Cuthbertson et al. ('40) based upon spectroscopic estimation of E in the tissues, urine and feces of the rat, it appears that the tocopherol molecule is either readily broken down by the body or is ineffectively absorbed and stored.

SUMMARY AND CONCLUSIONS

1. A total of 338 bio-assay tests on tissues of rats receiving approximately 4, 100 and 10,000 times the minimal daily requirement of vitamin E reaffirms its wide distribution in the animal body.

2. At the low level of intake, which is considered sub-optimal, the heart, lung and spleen possess almost twice as much vitamin E per gram of fresh tissue as the musculature, body fat and other visceral organs (kidney, testis, epididymis, prostate and seminal vesicle), and about four times as much as the liver.

3. At moderately high levels of intake the storage in the viscera, musculature and body fat is augmented three to four and one-half times; the heart, lung and spleen receiving more than other tissues, while that in the liver is increased about fourteen times. The mammary gland concentrates about twice as much E as the liver.

4. At excessively high levels of intake the musculature and liver, respectively, possess about 12 and 150 times the vitamin E storage occurring at the low level of intake. Liver content of the vitamin affords the most useful measure of the previous intake and storage.

5. Despite the wide distribution of vitamin E in the body of the rat, the estimated total storage represents but a small fraction of the vitamin ingested, confirming the suggestion of others that the tocopherol molecule is either ineffectively absorbed or readily broken down in the organism.

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HEAT PRODUCTION OF THE RABBIT AT 28°C. AS AFFECTED BY PREVIOUS ADAPTATION TO TEMPERATURES BETWEEN 10° AND 31°C.[1]

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Several investigators have shown that the level of the heat production of the rat and of other small animals measured at a given environmental temperature is dependent on the temperature at which these animals were previously living. Kayser ('39), Ring ('39), Gelineo ('35), Giaja and Gelineo ('31), and Schwabe and his colleagues ('38) have noted differences in the basal metabolic rates of rats according to the different temperature levels at which they had been living before measurement, and in some cases this difference amounted to over 20%. An influence of adaptation to previous environmental temperature has also been found with small birds by Kayser ('29), Dontcheff and Kayser ('34), Gelineo ('34), and others. It is of interest to determine whether this effect of adaptation is confined to small animals or is true also of larger animals. During an investigation of the basal metabolic rate of rabbits and the factors affecting this rate, it became evident that the environmental temperature at which the rabbits had previously been living influenced their metabolism, despite a 24-hour period of adjustment at thermic neutrality prior to measurement. Hence a study was made to determine how the rabbit's metabolism measured at 28° is affected by the previous environmental temperature and by the length of stay at that temperature.

[1] A preliminary report of this study was presented on April 18, 1941, at the Chicago meeting of the American Physiological Society. (Am. J. Physiol., vol. 133, p. P360, 1941.)

PROCEDURE

Twelve adult rabbits of both sexes were used, including Dutch, Chinchilla, and New Zealand White breeds ranging in weight from 1.9 to 5.3 kg. Group I consisted of five rabbits whose average weight was 3.99 kg., and group II of seven other rabbits whose average weight was 3.24 kg. The average weight of the animals in each group did not change significantly throughout the experimental period.

An open-circuit system, employing the Carpenter gas-analysis apparatus, was used for the metabolic measurements,[2] as in our previous work on rabbits (Lee, '39 a). The first two measurements on any rabbit have been discarded, because the rabbit had not become adjusted to the experimental procedure. Only periods of complete repose have been used.

The animals lived for a stated period at a definite temperature level between 10° and 31°C. The average variation in daily temperature during this period did not exceed 3°C. at any environment except as noted for group I, series 5. The rabbit was then kept at 28° for 24 hours, without food, and immediately thereafter its metabolism was measured at 28° to 29°C. To establish the effects of living at these several temperatures on the metabolism measured at 28°, from three to five experiments were carried out with each rabbit following each period of adaptation. The rabbits in group I, in all the series except no. 2, had been living at a definite temperature for 1 week or more before measurements were made. Those in group II in all instances lived at the given temperature level for 3 to 4 weeks before the measurements. In each group the series were consecutive. The length of each series, as recorded in table 1, includes the period of adaptation and the period of metabolism measurements.

The results were calculated as total heat production per 24 hours, but because of differences in the individual weights of the rabbits from series to series, it was not considered correct to use these measured total heat values to estimate

[2] The author wishes to acknowledge the technical assistance of Mr. George Lee.

the effect of previous environmental temperature on the metabolism. Instead, the predicted basal total heat production of each rabbit was calculated from its average weight in each series by using the formula:

$$\text{Total heat production per 24 hours} = 40W + 20$$

in which W represents the weight in kilograms (Lee, '39 b). Calculation was then made of the percentage deviation of each rabbit's measured total heat production in each series from its predicted basal heat production for that series. These individual percentage deviations in each series were then compared with the percentage deviations from prediction noted in series 3, for with both groups the measurements in series 3 were representative of the basal metabolism. The percentage deviation from prediction found for each rabbit in series 3 was therefore used as a zero point to calculate, in the other series, the total percentage change in metabolism from the basal level. The individual total percentage changes for the several rabbits in each series were then averaged. It is believed that this treatment of the data corrects for any role that might have been played by the small individual differences in body weight. An example of the method of calculation follows.

Rabbit no. R-1 (group II)

SERIES NO.	WEIGHT	TOTAL HEAT PRODUCTION PER 24 HOURS, CAL.		CHANGE FROM PREDICTION
		Measured	Predicted basal	
	kg.			%
1	2.14	121.7	105.6	+15.3
3	2.47	112.7	118.8	— 5.1

$$\text{True change from basal level} = \frac{115.3}{94.9} = +21.5\%.$$

DISCUSSION OF RESULTS

In group I the rabbits first lived at 17° for 7 weeks (series 1, table 1), during the last four of which they were measured weekly. Under these conditions the heat production of the individual rabbits, measured at 28°, was respectively, 11.6, 20.2, 35.1, 17.1, and 11.1% above their baseline levels in series

3 and averaged 19.0% above the baseline. The third and fifth rabbits in this group had basal metabolic rates significantly below the prediction, yet their reactions to changes in environmental temperature were similar to those of the other rabbits.

Following series 1 the rabbits were shifted to an environment of 29° and measured on the second, fourth, seventh, and fourteenth days of stay at this temperature (series 2). The levels on the fourteenth day averaged 1.8% above the baseline.

TABLE 1

Metabolism of rabbits in group I, measured at 28°C.

SERIES NO.	LENGTH OF SERIES	PREVIOUS TEMPERATURE (AVERAGE)	MEASUREMENT	PER CENT CHANGE FROM BASELINE IN SERIES 3[1]
	weeks	*°C.*		
1	7	17	Average	+19.0
2	2	29	Average	+ 5.5
			14th day	+ 1.8
3	3	29	Average	± 0.0
			30th day at 29°	— 2.2
4	4	29	Average	— 4.3
			9th week at 29°	— 5.5
5	3	29	Average	— 1.6
			(except 2 hours daily at 17°C.)	
6	3	18	Average	+ 4.0
7	4	23	Average	+ 0.4
8	4	25	Average	— 1.5
9	7	23	None	—
10	7	28	Average	— 1.7
11	3	31	Average	— 7.1
12	9	22	Average	+ 3.1

Metabolism of rabbits in group II, measured at 28°C.

SERIES NO.	LENGTH OF SERIES	PREVIOUS TEMPERATURE (AVERAGE)	MEASUREMENT	PER CENT CHANGE FROM BASELINE IN SERIES 3[1]
1	9	10	Average	+17.0
2	6	22	Average	+ 5.3
3	8	29	4th week	+ 0.8
			Average	± 0.0
			8th week	— 1.5
4	7	23	Average	+ 4.2

[1] See explanation on page 85.

The measurements during the next 3 weeks (series 3) were obtained under conditions that conform to the prerequisites for basal metabolism measurements (Lee, '39 a). The percentage changes from baseline on the thirtieth day at 29° (series 3) were slightly lower than the average for the entire series, indicating that the heat production was still decreasing, although the major change occurred in the first 2 weeks. A further small decrease occurred on keeping the animals at 29° for another 4 weeks (series 4), and the values for the last week (ninth week at 29°) were slightly below the average for the series, showing that the animals were still adjusting themselves to the temperature.

To determine the effect of increasing the daily range of environmental temperature, the rabbits, still maintained at 29°, were placed for 2 hours each day at 17°C. over a period of 3 weeks (series 5). Under these conditions the heat production was slightly increased above that in the previous series. Next the group was placed at 18° for 3 weeks, with the result that the metabolism was noticeably increased. It did not, however, approach the initial level at 17°, for the stay at 18° following the long period of adaptation at 29° was too short to permit complete readjustment to the former level.

The temperature was then raised in small steps (series 7 to 11). This produced a consistent decrease in metabolism except in rabbits in series 10, which was preceded by a 7-week period (series 9) in which the temperature averaged 2° lower than in the previous series. There were no measurements during this period. The increase in temperature in series 10 resulted in a metabolic level close to that in series 8.

The minimum metabolism was found when the rabbits lived at 31° for a 3-week period (series 11), after a long, gradual approach to this temperature. This minimum level was 7.1% below the baseline in series 3. In series 12 the rabbits were kept at 22°, and the heat production increased markedly.

The differences in the temperatures at which the rabbits lived before measurement (17° to 31°C.) caused the heat

production, always measured at 28°, to show a maximum range of 26.1% in the deviations from the baseline.

It appears that the adjustment of the 28° metabolism to an increase in temperature took place largely in a short period of time, whereas the adjustment to a decrease in temperature did not take place so rapidly.

Further experiments in later series were complicated both by reduction in the number of rabbits in group I and by other features. The general trends indicated by these experiments were that when the temperature was maintained at 15° for 8 weeks, the heat production increased markedly, and that prolongation of the stay at 15° for 5 weeks more resulted in a further increase in heat production, which approached the initial level at 17°C. When, in the final series, the temperature was raised to 23°, the metabolic level decreased again.

The same trends of metabolic reaction to previous environmental temperature were shown by group II (table 1). The rabbits in this group were adapted initially to 10° for a period of 9 weeks. Here again as the temperature of the environment was increased, the metabolism measured at 28° decreased. The first measurements in series 3 were made after the rabbits had been at 29° for 4 weeks, and these measurements averaged 0.8% above the average baseline for this series. Measured in the eighth week at this temperature, the metabolic level was 1.5% below the average baseline. This comparison of the initial and final measurements in the same series after an interval of 4 weeks brings out the same picture as shown by group I, namely, that there is a further decrease in heat production as the stay at thermic neutrality is prolonged, at least up to 8 weeks. When the previous temperature was lowered again (series 4), the metabolic level increased. The maximum changes found in metabolic level were, for the individual rabbits, 29.9, 21.5, 7.5, 15.6, 17.8, 15.9, and 11.0% above the baseline and averaged +17%.

In 89% of eighty cases in both groups in which the previous temperature was changed from one level to another or was maintained for a longer period at the same level, it was noted,

respectively, that the metabolism varied inversely with the direction of the change in temperature or additional change in metabolism took place in the direction previously noted.

The maximum differences found in groups I and II between the heat production resulting from adaptation to temperatures of 17° and 29° and of 10° and 29°, respectively, are in line with the difference noted by Ring with rats whose metabolism, measured at thermic neutrality after they had lived at 3°, was increased 21% above the basal metabolism when they had been at 30°C. (thermic neutrality).

In an earlier paper describing the prerequisite conditions for measurement of the basal metabolism of the adult rabbit, it was stated that "the rabbits should have been living at environmental temperatures above 20° but not above 32°." Many of the measurements in both group I and group II were made under these requirements for basal metabolism measurements, and except for series 11, group I, the values are within ±5.5% of the average series value taken as the baseline. Even within the limits of 20° to 32°C. the metabolic level can be measurably influenced by changes in previous environmental temperature. Certainly in studies in which changes in basal metabolism are used as measures of the effects of superimposed conditions, the environmental temperature should be maintained at a given level between 20° and 32°C., preferably 28° to 29°, and the rabbit should be habituated to the temperature for 3 weeks prior to the measurements. The effect of previous environmental temperature offers a partial explanation of the wide variations noted in the literature in the so-called "basal metabolism measurements" on rabbits.

SUMMARY

The level of the metabolism of twelve adult rabbits, in repose, at 28°, measured after they had been kept at 28° and without food for 24 hours, was dependent on the temperature at which they had been living prior to this period and the length of time they had been at this temperature. The major metabolic adjustment to an increase in temperature toward

thermic neutrality took place in 2 to 3 weeks, but further adjustment continued up to at least 2 months. The adjustment to lower temperatures was less rapid. The maximum range in the deviations from the basal level in the metabolism measured at 28° amounted to 26.1% in a group of five rabbits that had lived at 17° and subsequently at 31°, and to 17.0% in a group of seven rabbits that had lived at 10° and later at 29°C. In general, the metabolism varied inversely with the change in previous temperature, 89% of eighty observations conforming to this finding. Prolongation of the stay at a given temperature caused a further change in metabolism in the same direction. When basal metabolism measurements on the rabbit are to be used as a measure of the effect of a superimposed condition, the previous environmental temperature should be maintained at 28° to 29°C., and the rabbit should be habituated to this temperature for 3 weeks prior to the measurements.

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CHOLINE METABOLISM

VIII. THE RELATION OF CYSTINE AND OF METHIONINE TO THE REQUIREMENT OF CHOLINE IN YOUNG RATS [1]

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FOUR FIGURES

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A supplement of cystine in a choline-deficient diet increases both the deposition of liver fat in older rats (Beeston and Channon, '36) and the incidence and severity of hemorrhagic degeneration in young rats (Griffith and Wade, '40). Griffith ('41) found no direct relation between the level of the cystine supplement and the degree of the cystine effect and suggested that the observed increase in the requirement of choline was due to an improved nutritional state rather than to some direct antagonism of cystine and choline. Although cystine was recognized as the limiting essential amino acid in casein following the classical experiments of Osborne and Mendel ('15), emphasis was shifted to the indispensability of methionine rather than of cystine by the observations of Womack, Kemmerer and Rose ('37). However, Rose ('37) pointed out that supplementary cystine greatly improves a diet which contains insufficient methionine for the total sulphur requirement. Rations containing casein as the protein are occasionally fortified with cystine (Mackenzie et al., '39) but this practice has not been general. The present paper describes experiments which are interpreted as demonstrating

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that the 18% casein diet is a suboptimal source of cystine and that supplementary cystine improves the nutritional level and thereby indirectly increases the requirement of choline. Evidence is also presented for the conclusion that methionine which serves as a source of labile methyl (du Vigneaud et al., '40; Griffith and Mulford, '41) does not serve as a source of cystine sulphur.

EXPERIMENTAL

Young male rats, 21 to 26 days of age and 38 to 42 gm. in weight, were used and were equally distributed in the various groups according to age. The appearance of the kidneys was noted at the end of the experimental period and the per cent of animals in each group showing the hemorrhagic state (Griffith, '40) is recorded in figures 2, 3 and 4. The term liver fat in figures 1 to 4 refers to the total chloroform-soluble substances of the liver. The distance between the tip of the nose and the anus was measured and is recorded in figure 1 and in tables 1 and 2 as body length.

The low choline diet, AC 50, consisted of purified casein 18%, lard 19, sucrose 49, salt mixture 4 (Hawk and Oser, '31), calcium carbonate 1, agar 2, powdered yeast 6 [2] and fortified cod liver oil 1%. All supplements of choline and cystine and other additions to the basal diet replaced an equal weight of sucrose.

RESULTS AND DISCUSSION

Effect of a supplement of cystine in an 18% casein diet. The effect of the addition of cystine to diet AC 50 plus various levels of choline during 20- or 40-day periods is shown in figure 1 and in tables 1 and 2. In every case, the rats receiving the extra cystine were heavier and longer and these gains were accomplished with little or no increase in the consumption of food, the gain in weight per gram of food during a 20-day period averaging 0.55 gm. on the basal diet and 0.60 to 0.62 gm. on the supplemented diet. The more

[2] Anheuser-Busch, strain G.

efficient utilization of food is evident during the first but not during the second 20-day period (table 1), a finding which suggests a greater need of cystine in young rats 3 to 6 or 7 weeks of age. The beneficial effect of the extra cystine is emphasized by the difference in the weights of groups 2 and 6 (table 1) after 150 days on the basal and supplemented diets (data not shown in table 1). At the end of this period the rats in group 2 averaged 286 gm. in weight, seven weighing

TABLE 1

The effect of supplements of cystine in an 18% casein diet (AC 50) on growth and utilization of food in 40-gm. male rats during a 40-day experimental period.

GROUP	1	2	3	4	5	6	7	8[1]
No. of rats in group	40	20	20	20	20	20	38	20
Dietary casein, %	18	18	18	18	18	18	18	15
Cystine supplement, %	0	0	0.04	0.08	0.3	0.5	0.3	0.31
Choline chloride supplement, %	0.5	0.3	0.3	0.3	0.3	0.3	0.5	0.3
Gain in weight, gm.								
20 days	77	79	88	93	87	91	84	91
40 days	155	158	174	177	172	176	165	177
Body length, cm.								
20 days	17.7	17.8	18.1	18.4	18.2	18.4	18.2	18.4
40 days	20.8	21.2	21.2	21.4	21.5	21.8	21.2	21.8
Gain in weight per gram of food, gm.								
0 to 20 days	0.56	0.54	0.56	0.59	0.64	0.63	0.61	0.60
0 to 40 days	0.46	0.46	0.47	0.47	0.50	0.49	0.47	0.49
20 to 40 days	0.39	0.39	0.40	0.39	0.41	0.40	0.38	0.40

[1] 0.09% dl-methionine added to diet.

less than 270 gm. and five weighing more than 300 gm.; in contrast, the rats in group 6 averaged 312 gm. in weight, none weighing less than 270 gm. and eleven weighing more than 300 gm.

A 15% level of casein, supplemented with both methionine and cystine, permitted normal growth during a 40-day period (group 8, table 1) and methionine alone satisfactorily replaced the cystine supplement in the 18% casein diet during a

20-day period (group 6, table 2). The addition to the basal diet of 4% of lactalbumin (group 8, table 2) also satisfied the requirement of sulphur amino acids but the 22% casein diet appeared less efficient (group 7, table 2). Growth in these experiments was not limited by a deficiency in vitamins of the B complex because no additional improvement followed an increase in the level of yeast from 6% to 10% (group 9, table 2). All of the rats listed in table 2 received a supplement of 5 mg. of α-tocopherol on the tenth day. The results

TABLE 2

The effect of supplements of cystine, methionine, yeast and protein in an 18% casein diet (AC 50 + 0.3% choline chloride) on growth and utilization of food in 40-gm. male rats during a 20-day experimental period.

GROUP	1	2	3	4	5	6[1]	7[2]	8[3]	9[4]
No. of rats in group	24	23	24	23	23	24	22	24	22
Cystine supplement, %	0	0.1	0.2	0.3	0.5	0	0	0	0
Gain in weight, gm.	71	77	80	80	80	81	75	81	83
Body length, cm.	17.6	17.9	18.2	18.2	18.2	18.2	17.9	18.2	18.2
Gain in weight per gram of food, gm.	0.55	0.58	0.62	0.62	0.63	0.61	0.58	0.60	0.60
Dietary sulphur, %[5]	0.134	0.161	0.188	0.214	0.269	0.214	0.164	0.190	0.214

[1] 0.362% dl-methionine added to diet.
[2] 4% casein added to diet.
[3] 4% lactalbumin added to diet.
[4] 4% yeast added to diet.
[5] Calculated from analyses reported by Baernstein ('36). Sulphur of yeast not included in total.

with groups 3 and 4 (table 1) and with groups 2 and 3 (table 2) indicate that the 18% casein diet requires 0.1 to 0.2% of additional cystine to permit optimum growth and utilization of food.

Relation of dietary cystine and choline to choline deficiency. The effect of cystine and of choline on liver fat is illustrated in figure 1 which shows the increase in liver fat due to a cystine supplement in low choline diets during a 20-day period. An adequate level of choline prevents this action of cystine

but does not influence appreciably its effect on growth and on utilization of food. Figure 2 shows the effect of three levels of choline and of six levels of cystine on liver fat and on renal lesions during an 8-day period. The deposition of liver

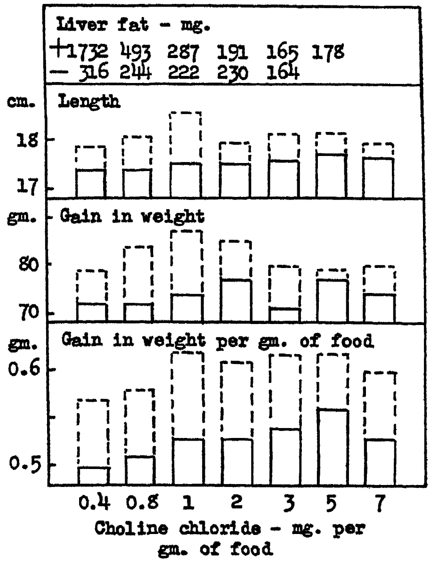

Fig. 1 Relation of supplementary choline and cystine (0.3%) in an 18% casein diet (AC 50) to the deposition of liver fat, to growth and to the utilization of food in 40-gm. male rats during a 20-day period. The groups receiving cystine are indicated by the broken lines. Each group consisted of twenty or more rats.

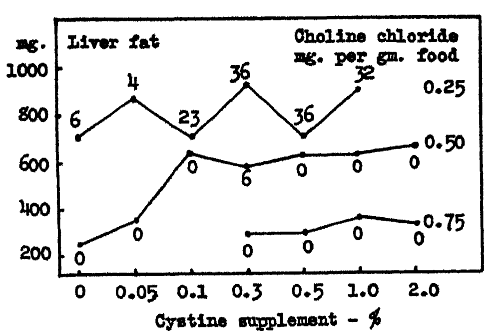

Fig. 2 The relation of the level of a supplement of cystine to the deposition of liver fat and to the incidence of renal lesions in 40-gm. male rats during an 8-day period. The numbers on the curves show the per cent of animals with renal lesions. Each group consisted of twenty or more rats.

fat was variable with 0.25 mg. of choline per gram of food, a result which is explained by the poor nutritional state of such animals (Griffith and Wade, '40). Groups fed rations containing 0.5 to 0.75 mg. of choline per gram of food showed no effect of the level of the cystine supplement provided 0.1% or more of cystine was added. These results extend and confirm the previous observation (Griffith, '41) that the minimum daily requirement of choline for the protection of the kidneys is the same on diets containing 0.3% or 1.0% of added cystine.

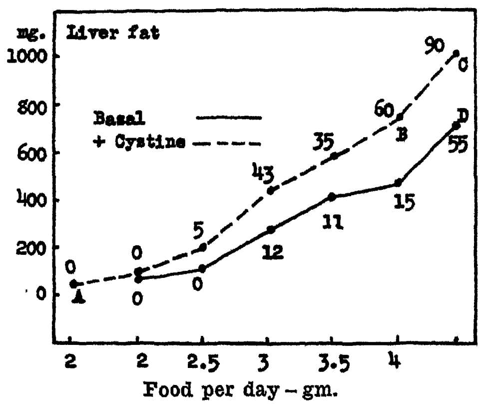

Fig. 3 The relation of restriction of the consumption of diet AC 50, with and without a supplement of 0.5% of cystine, on the deposition of liver fat and on the incidence of renal lesions in 40-gm. male rats during an 8-day period. The numbers on the curves show the per cent of animals with renal lesions. Group A received a special diet which contained in 2 gm. the same amount of each constituent except lard and sucrose that was present in 4 gm. of AC 50 + 0.5% cystine (group B). Groups C and D consumed the experimental diets ad libitum, the average daily food intakes being 4.6 and 4.9 gm., respectively. Each group consisted of twenty or more rats.

It is significant that a supplement of 0.1% of cystine in the 18% casein diet produced the maximum deposition of liver fat as well as nearly optimum growth and utilization of food (tables 1 and 2).

Griffith and Mulford ('41) found that the choline requirement is decreased by restriction of the intake of a food which is injurious if fed ad libitum. The comparison in figure 3 of the effect of restriction of the basal diet, with and without

a supplement of cystine, demonstrates that the signs of choline deficiency are increased both by the cystine supplement and by the ingestion of larger quantities of food. Of particular interest is the result with group A which was fed 2 gm. daily of a food mixture containing the same amount of each dietary constituent, except lard and sucrose, that was present in 4 gm. of the food supplied group B. Group A consumed 0.5 gm. of lard and 0.2 gm. of sucrose and group B consumed 0.8 gm. of lard and 1.94 gm. of sucrose daily. Therefore, group A, with no signs of choline deficiency, and group B, with a high incidence of renal lesions and with markedly fatty livers, consumed rations differing mainly in caloric value.

It seems logical to conclude that the increased need of choline in young rats on an 18% casein diet supplemented with cystine is due to the stimulation of metabolism resulting from the addition of required cystine to a cystine-deficient food mixture. No explanation is thus necessary for the failure of excess cystine to affect the choline requirement. There is no evidence in these experiments that cystine is toxic per se. It is entirely possible that the administration of an excess of cystine may result in injury to the tissues of the rat (Curtis et al., '27; Lillie, '32; Earle and Victor, '41). However, the present study suggests that due consideration be given the character of a food mixture before it is concluded that an effect of a cystine supplement is directly related to the cystine rather than to a stimulation of metabolism which in turn brings to light previously unrecognized deficiencies. On the other hand it is pertinent that a supplement of cystine offsets the toxicity of certain injurious compounds (White and Jackson, '35; Stekol, '37; White and White, '39) and halves the time required for the action of an anti-gray hair factor (Pavcek and Baum, '41).

Relation of dietary cystine and casein to choline deficiency. It became of interest to determine at which level of dietary casein the effect of the cystine supplement is no longer evident. Figure 4 shows that in the absence of choline 30% of casein is required to supply enough methionine to prevent signs of

choline deficiency. This is also the level at which the effect of added cystine disappears. In other words, a diet containing 26% of casein appears deficient in both methionine (labile methyl) and in cystine in spite of the fact that this diet contains as much total sulphur (0.194%) in the form of methionine and cystine as is present in the adequate choline-supplemented 18% casein diets to which additional cystine or lactalbumin is added (groups 3 and 8, table 2). It is therefore concluded that, if methionine is used as a source of methyl for the

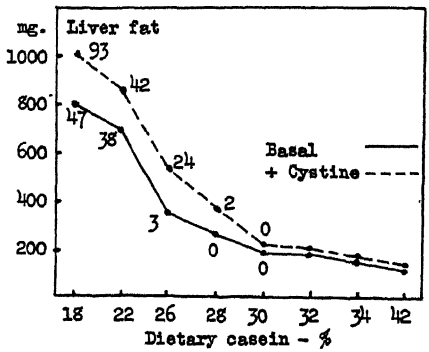

Fig. 4 The relation of supplementary cystine (0.3%) and of dietary casein (18 to 42%) to the deposition of liver fat and to the incidence of renal lesions in 40-gm. male rats during an 8-day period. Casein above 18% replaced an equal weight of sucrose in the basal diet, AC 50. The numbers on the curves show the per cent of animals with renal lesions. Each group consisted of twenty or more rats.

synthesis of choline, its sulphur is not available for the formation of cystine. Such a conclusion is not compatible with the demethylation of methionine to labile methyl and homocysteine if a direct conversion of homocystine to cystine occurs (du Vigneaud et al., '33). It is in agreement with the idea expressed by Toennies ('40) that homocysteine must first be converted to methionine before its sulphur is used as cystine sulphur. This conversion would not be possible on the 26% casein diet, unless supplementary choline or some other source of labile methyl is added, because this diet is deficient in labile methyl.

SUMMARY

1. Supplementary cystine (0.1 to 0.2%) or methionine improves the growth and efficiency of utilization of food in young rats on an 18% casein diet.

2. The deposition of liver fat and the incidence of renal hemorrhagic degeneration in young rats on a low choline diet are increased by supplements of cystine (0.1 to 0.2%) which improve the nutritive value of the diet. Larger supplements of cystine have no effect on the requirement of choline.

3. In the absence of dietary choline, 30% of casein is required to supply sufficient methionine and cystine for the prevention of signs of choline and cystine deficiency.

4. Evidence is presented for the conclusion that methionine which is needed and used as a source of labile methyl is not utilizable as a source of cystine sulphur.

ACKNOWLEDGMENT

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THE EFFECT OF ADDED GLUCOSE UPON THE DIGESTIBILITY OF PROTEIN AND OF FIBER IN RATIONS FOR SHEEP [1]

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During a recent investigation in this laboratory (Mitchell and Hamilton, '40) concerned with the utilization of the energy of rations containing varying percentages of protein and of glucose when fed to beef calves, it was found that increasing the gross energy of rations by about 30% by the addition of glucose failed to increase their metabolizable energy contents (expressed in calories per kilogram of dry matter consumed). When the metabolizable and net energy values of glucose were calculated by the usual indirect procedure, these values were found to be considerably lower than might be expected. The average of twelve estimates indicated that glucose [2], with a gross energy value of 3680 calories per kilogram of dry matter, possessed on the same basis 2401 calories of metabolizable and 1175 calories of net energy when fed to beef calves.

Similar low metabolizable and net energy values for starch, when fed to steers, have been obtained by Armsby and Fries ('18) and by Kellner and Köhler ('00).

The failure to increase the metabolizable energy value of a ration, when the proportion of the so-called available carbohydrate fraction is increased over the so-called non-available carbohydrate fraction, at first may seem paradoxical. How-

[1] Aided by the Works Progress Administration, Project No. 80057.

[2] Cerelose. Kindly supplied by The Corn Products Refining Company.

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ever, it is a well-known fact that the addition of starch or molasses to a ration for ruminants usually decreases the digestibility of the nutrients in the basal ration, this being particularly noticeable in case of protein and of crude fiber (cf. Kellner, '09; Lindsey and Smith, '10; Ewing and Wells, '15; Armsby and Fries, '18; Briggs, '37; and Scharrer and Nebelsiek, '38). However, some data have been interpreted to indicate a more favorable effect of soluble sugars added to rations for ruminants (Patterson and Outwater, '07, and Williams, '25).

In four trials with beef calves, Mitchell and Hamilton ('40) found that corn sugar, added to rations containing varying percentages of protein, decreased the digestibility of the crude fiber by about 25%. In order to test more completely the effects of adding corn sugar to rations when fed to ruminants, the experiments described below were carried out.

PLAN OF EXPERIMENT

The plan of the experiment was to determine the digestibility of the nutrients and the metabolizable energy in rations with and without added glucose. Six cross-bred ewe lambs, weighing between 60 and 100 pounds, were used as experimental animals. The rations used were of the same composition as those fed to steers in a previous experiment (Mitchell and Hamilton, '40). The basal ration consisted of cut timothy hay, ground yellow corn, and cottonseed meal in the approximate ratios of 2:2:1. The ration containing the glucose, hereafter referred to as the sugar ration, was the same as the basal except that glucose was added to the extent of 20 to 30% — the exact amount of sugar added depended upon the avidity with which the individual sheep ate the ration with the added sugar. The plan of feeding involved a double reversal procedure. The basal ration was fed at a level calculated to just maintain live weight. The food maintenance requirement for these sheep was assumed to be adequately covered by 1.64 pounds of this particular ration per 100 pounds live weight. When receiving the sugar rations, each sheep

received exactly the same amount of the basal ration as she did in the no-sugar period plus 150 to 200 gm. of the corn sugar daily. In addition, in each period each sheep received daily 6 gm. bone meal, 3 gm. yeast, 3 gm. salt, and 0.5 cc. cod liver oil. While six sheep were used and the plan was to carry out a double reversal procedure on each, it was possible to obtain such data on but four of the animals. In one case data were obtained on the basal ration in one period and on the sugar ration in one period. In another case the sheep would not eat the basal ration so data were obtained on the sugar ration for one period only.

The apparatus consisted of six digestion crates which have been used in this laboratory for a number of years (cf. Keilholz, '26). Daily collections of urine and feces were made for 10 days and each collection period was preceded by a 7-day preliminary feeding period. Feces samples were preserved by drying at 50 to 60° C. and urine samples were preserved with toluene. Methods of analysis were those of the A. O. A. C. ('40). Gross energy of feeds, feces, and urines was determined by a Parr Oxygen bomb calorimeter. The urine samples were evaporated on cellulose blocks before burning. The metabolizable energy of the rations was calculated by subtracting, from the gross energy consumed, the sum of the gross energy of the feces, urine, and methane.[3] The energy in the urine was corrected for nitrogen balance according to Armsby's ('17, p. 641) procedure, using Rubner's (1885) factor of 7.45 Calories for each gram of nitrogen balance.

RESULTS

The essential collection data are tabulated in table 1. The average composition of the basal and of the sugar rations are, respectively, 91.6 and 91.7% dry matter, 14.6 and 13.1% crude protein, 15.5 and 11.8% crude fiber, 53.0 and 62.1% nitrogen-

[3] The methane was calculated by the use of Armsby's factor of 4.5 gm. of methane per 100 gm. of digestible carbohydrates. This factor may not be strictly applicable to sheep, but, since the rations were similar in most respects, the errors probably are not serious.

TABLE 1

Collection data.

SHEEP	BREED	WEIGHT		DAILY FEED[1]				AVERAGE DAILY ORTS	EXCRETED DAILY		PERIOD
		Start	End	Timothy hay	Corn	Cotton-seed meal	Sugar		Feces (dry)	Urine and washings	
		lb.	*lb.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
554	Hampshire Cross	57.0	58.5	182	182	102	....	....	149	4009	I[3]
537	" "	73.0	74.5	224	224	126	....	....	181	2357	"
822	Southdown Cross	91.5	94.5	264	264	148	....	....	216	2647	"
882	" "	75.0	76.5	224	224	126	....	....	181	2466	"
805[2]	" "	79.5	78.0	238	238	134	....	161	165	2395	"
554	Hampshire Cross	62.5	63.0	182	182	102	150	....	191	4027	II[4]
537	" "	80.5	81.5	224	224	126	200	....	237	2621	"
540[2]	" "	80.0	80.5	184	184	104	200	33	174	2690	"
822	Southdown Cross	98.0	100.5	264	264	148	200	....	256	2880	"
882	" "	80.0	83.0	224	224	126	200	....	211	2627	"
805[2]	" "	81.0	82.5	238	238	134	150	94	246	2584	"
554	Hampshire Cross	61.5	62.0	182	182	102	....	....	150	4616	III[5]
537	" "	80.5	81.0	224	224	126	....	....	182	3024	"
822	Southdown Cross	99.5	99.5	264	264	148	....	....	209	3183	"
882	" "	81.5	82.0	224	224	126	....	....	178	2652	"

[1] Each sheep was fed at the daily rate of 1.64 pounds per 100 pounds live weight. In addition to the experimental ration each sheep received daily 6 gm. bone meal, 3 gm. yeast, 3 gm. salt, and 1 cc. cod liver oil.

[2] Orts in this case. Nutrients in orts accounted for.

[3] Period I — January 30 to February 8.

[4] Period II — February 20 to March 1.

[5] Period III — March 8 to March 17.

free extract, 4.2 and 3.4% ether extract, 4.3 and 3.3% ash, and 4.13 and 3.94 cals. of gross energy per gram. The apparent digestion coefficients, the total digestible nutrients, and the metabolizable energy contents of the two rations are tabulated in table 2. According to these data, the addition of sugar increased the apparent digestibility of dry substance, N-free extract, and total carbohydrates, but decreased the apparent digestibility of total nitrogen and crude fiber as well as the metabolizable energy content.

Perhaps a better way of assessing the significance of these differences is to compare the values obtained on each sheep and with the animals consuming the two different rations. This is possible in five of the six sheep used. In four cases the double reversal procedure was used and in these cases the two basal periods were averaged and this average compared with the sugar period. In one case there are data on one basal and one sugar period only. These comparisons are susceptible to statistical analysis of paired data according to Student's ('08) method. In table 3 these paired comparisons are summarized and the results of the statistical treatment are given. The P values or the odds given indicate the probability that chance alone caused differences as great as those obtained. Assuming that P values of 0.03 or less, or odds of 1:33 or more, are significant, then the following conclusions may be made with reference to the effect of the addition of corn sugar to the basal ration: (1) It caused an increase in the apparent digestibility of the dry matter, N-free extract, and total carbohydrates. (2) It caused a decrease in the apparent digestibility of total nitrogen and crude fiber, and a decrease in the metabolizable energy concentration. (3) The sugar ration contained an average of 1.7 pounds of T. D. N. more per 100 pounds than did the basal ration. The probability that this difference was fortuitous was 0.0307, a value that most would consider significant. (4) It caused no difference in the apparent digestibility of either ether extract or gross energy.

TABLE 2

Coefficients of apparent digestibility by individual sheep.

SHEEP	RATION	PERIOD	DRY SUBSTANCE	TOTAL NITROGEN	CRUDE FIBER	ETHER EXTRACT	N-FREE EXTRACT	TOTAL CARBO-HYDRATES	GROSS ENERGY	TOTAL DIGESTIBLE NUTRIENTS	METABOLIZABLE ENERGY
			%	%	%	%	%	%	%	%	Cal. per kg. Dry matter
554 H	Basal	I	66.2	62.4	48.2	69.2	77.0	70.4	65.7	63.7	2417
	Basal + sugar	II	67.7	56.9	28.7	73.5	79.0	70.9	65.6	64.5	2311
	Basal	III	66.4	61.2	45.8	72.1	76.8	69.9	65.9	63.8	2464
805 S	Basal	I	59.9	53.8	36.5	73.8	73.7	65.1	62.8	58.5	2363
	Basal + sugar	II	61.1	41.0	29.9	68.8	75.2	67.2	60.0	59.0	2136
882 S	Basal	I	66.6	67.0	44.1	75.3	76.7	69.2	66.9	64.1	2547
	Basal + sugar	II	71.9	58.5	37.7	79.2	83.1	76.0	70.2	68.9	2487
	Basal	III	67.7	63.6	49.1	76.7	78.2	71.7	68.3	65.7	2568
537 H	Basal	I	66.6	66.1	40.6	80.5	76.3	68.0	66.5	63.7	2476
	Basal + sugar	II	68.3	56.6	26.0	69.0	80.1	71.6	65.0	64.7	2262
	Basal	III	67.0	63.6	42.9	74.1	76.6	69.1	66.5	63.7	2579
822 S	Basal	I	66.1	65.9	44.3	74.9	76.7	69.2	66.7	63.9	2472
	Basal + sugar	II	69.7	57.4	37.4	76.0	81.1	73.9	67.9	67.0	2456
	Basal	III	67.6	61.4	50.2	72.8	78.1	71.9	67.9	65.2	2557
540 H	Basal + sugar	II	71.9	53.1	36.5	74.8	82.6	76.1	69.0	67.8	2381
	Average–Basal	(9)	66.0	62.8	44.6	74.4	76.7	69.4	66.4	63.6	2494
	Average–Basal + sugar	(6)	68.4	53.9	32.7	73.6	80.2	72.6	66.3	65.3	2339

Since each sheep, when on the sugar ration, consumed the same, or very nearly the same, amount of the basal ration as she did when on the basal ration alone, any change in the digestibility of the nutrients in the sugar ration may be attributed directly to the added sugar. Thus by assuming that the added sugar was completely digested and that the digestibility of the nutrients in the basal ration remained unchanged by this addition, the total nutrients that should have been digested from the sugar ration may be calculated. These cal-

TABLE 3

Summary and statistical analysis of coefficients when each sheep serves as her own control.

	AVERAGES FOR BASAL RATION	AVERAGES FOR BASAL + SUGAR RATION	DIFFERENCE IN FAVOR OF BASAL + SUGAR RATION	STANDARD DEVIATION	P VALUE [1]	ODDS
Dry matter, pct.	65.4	67.7	+2.36	1.36	0.0123	1: 79
Total nitrogen, pct.	61.9	54.1	—7.78	2.69	0.0023	1: 435
Crude fiber, pct.	43.8	31.9	—11.86	1.25	0.0001	1: 10,000
Ether extract, pct.	74.3	73.3	—1.00	1.49	0.127	1: 8
N-free extract, pct.	76.4	79.7	+3.34	1.46	0.005	1: 200
Total carbohydrates, pct.	68.9	71.9	+3.00	1.59	0.0022	1: 454
Gross energy, pct.	66.0	65.7	—0.26	0.23	0.335	1: 3
Total digestible nutrients, pct.	63.1	64.8	+1.70	1.34	0.0307	1: 33
Metabolizable energy, Cals. per kg. dry matter	2480	2330	—1.50	82.5	0.010	1: 100

[1] Probability or odds that the differences were fortuitous.

culations show that, on the assumption that the sugar had been completely digested, there was a decrease in the digestibility of all nutrients in the basal ration. These decreases amounted to 40.3 gm. of dry matter, 7.5 gm. crude protein, 10.5 gm. crude fiber, 0.2 gm. ether extract, 22.7 gm. of nitrogen-free extract, 33.4 gm. total carbohydrates, and 210 Calories of gross energy.

A decrease in digestibility of the energy and of the nitrogen-free extract could be accounted for by a decrease in the digestibility of these nutrients in either the basal part of the ration

or in the sugar or in both. However, the decrease in the digestibility of the nutrients not in the sugar, namely, protein, fiber and fat, can be accounted for only by a decrease in the digestibility of these nutrients in the basal part of the ration.

As Armsby ('17, p. 619) has suggested, a possible explanation for the decrease in the apparent digestibility of protein is the increase in metabolic nitrogen due to the added sugar. According to Harris and Mitchell ('41), sheep on a nearly nitrogen-free ration excrete 5.5 mg. of metabolic nitrogen in the feces per gram of dry food consumed. Using this factor, it may be calculated that the average true digestibility of the nitrogen of the basal ration was 81.8 and that of the basal plus sugar ration was 79.4. The difference of 2.4% was found to be not significant. Thus the significantly lower apparent digestibility of the nitrogen in sugar ration, as compared with that of the basal ration, is probably accounted for entirely by the sugar addition.

As to the marked decrease in the digestibility in the fiber in the basal ration when sugar is added, the most probable explanation seems to be that the microorganisms in the rumen, which, in the absence of a more soluble form of carbohydrates, attack the cellulose, prefer the sugar to the fiber. That this is apparently true is indicated from the following observations.

It may also be calculated that, if the sugar of the sugar ration had been completely absorbed and if the nutrients in the basal fraction of the sugar ration had been digested to the same extent as they were in the absence of sugar, 39.2 gm. of crude fiber and 393.9 gm. of nitrogen-free extract would have been digested, but actually only 28.7 gm. of crude fiber and 371.2 gm. of nitrogen-free extract were digested. There would have been 2159 Cals. of energy digested on the same assumptions and actually 1949 Cals. were digested. Furthermore, if it could be assumed that all of the sugar had been absorbed, as would be reasonable in the case of non-ruminants, and if the basal fraction of the sugar ration had had the same metabolizable energy content that it did when fed alone, it

may be calculated that the metabolizable energy content of the sugar ration would have been 2822 Cals. per kilogram of dry matter instead of 2339. Also the apparent digestibility of the total carbohydrates would have been 78.7 ($433.3 \div 550.7 \times 100$) instead of the actual value of 72.6%.

While the metabolizable energy content of the sugar ration was slightly, though definitely, less than that of the basal ration, the metabolizability of the gross energy of the two rations was almost identical, i.e., 54.8 and 54.2% of the gross energy of the basal and of the sugar rations, respectively, were metabolizable.

The indications in this investigation that glucose is readily fermented by the microorganisms of the paunch, are in harmony with the recent in vitro studies of Woodman and Evans ('38) and the in vivo studies of Nikitin ('39).

SUMMARY

Using six sheep as experimental animals and the double reversal method of feeding, the effect of corn sugar upon the digestibility of the nutrients of a ration was studied with the following results:

1. It increased the apparent digestibility of (a) dry matter, (b) N-free extract, and (c) total carbohydrates.

2. It decreased the apparent digestibility of (a) total nitrogen and (b) crude fiber; and decreased the metabolizable energy content.

3. It had no significant effect upon the apparent digestibility of either ether extract or gross energy.

4. The metabolizability of the energy of the sugar-free ration is approximately the same as that of the sugar-containing ration.

5. It had no effect upon the true digestibility of total nitrogen, since the apparent decrease in digestibility can be entirely accounted for by the estimated increase in metabolic nitrogen in the feces of the sheep on the sugar ration.

6. The rather marked decrease in the digestibility of the fiber when sugar was present was apparently due to the preference by the microorganisms of the paunch for sugar.

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EFFECT OF CONTROLLED ASCORBIC ACID INGESTION UPON URINARY EXCRETION AND PLASMA CONCENTRATION OF ASCORBIC ACID IN NORMAL ADULTS

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ONE FIGURE

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Inasmuch as the optimal intake of ascorbic acid has not yet been satisfactorily determined, it seemed desirable to make a further study of certain criteria for determining requirement, namely, urinary excretion of ascorbic acid and the concentration of ascorbic acid in blood plasma in relation to tissue saturation.

Few studies have been reported in which urinary excretion of ascorbic acid and the concentration of ascorbic acid in the blood have been determined in subjects on a controlled intake of vitamin C over long periods of time. In most cases these studies have been on subjects receiving diets deficient in vitamin C, and blood determinations have not been made daily (Van Eekelen, '36; Rietschel and Mensching, '39; Rietschel and Schick, '39; and Crandon, Lund and Dill, '40).

The study which is most nearly comparable with the one to be reported here is that of Ralli, Friedman and Sherry ('39). In general these investigators proceeded from lower to higher levels of intake as the study progressed. There were four

[1] The data in this paper are taken from the thesis presented by Clara A. Storvick to the faculty of the Graduate School of Cornell University in fulfillment of the requirement for the Ph.D. degree, June, 1941. Further details will be found in the thesis.

instances only in which a period on decreased intake followed a period in which the subjects' tissues were presumably saturated.

In the study which is reported here, the subjects were normal adults who were previously prepared by saturation of the tissues. They were given a basal diet which was low in vitamin C and supplemented, for each experimental period, with a constant level of synthetic ascorbic acid.[2] Urinary excretion and fasting plasma ascorbic acid values were determined daily. Inasmuch as the use of a test dose has proved valuable in indicating the state of tissue reserves, each period was terminated by a standard test dose. The data on the daily urinary excretion and plasma ascorbic acid were treated statistically to determine whether or not there was any correlation between these two values.

EXPERIMENTAL PROCEDURE

Experimental subjects

Two men and four women served as subjects in this study. They were all actively engaged in some type of nutrition research and were interested in and able to cooperate in every phase of the study. Physical examinations by physicians of the Medical Clinic at Cornell University indicated that they were in normal health. Data as to the sex, age, height, average weight, and the weight variation of the subjects during the course of the experiments are shown in table 1.

Methods

The experimental diet and the methods for the collection and preservation of urine and for the determination of ascorbic acid therein were the same as those reported previously (Belser, Hauck and Storvick, '39). The direct titration with indophenol does not rule out the effect of non-specific reducing substances, but the error due to this circumstance is probably small (Abbasy et al., '35; Belser, Hauck and Storvick, '39).

[2] Hoffman — LaRoche.

Since the levels of ascorbic acid used in this study were relatively high and the urinary excretions correspondingly high, it is improbable that the errors inherent in the method used are of sufficient significance to affect the reliability of the data reported.

Reduced ascorbic acid in plasma was determined by the micromethod of Farmer and Abt ('36) on fasting blood samples. If hemolysis or clotting occurred it was necessary to take a second sample of blood. In some cases this was done

TABLE 1

Sex, age, height, average weight, and weight variation of the experimental subjects.

SUBJECT	SEX	AGE [1]	HEIGHT	AVERAGE WEIGHT	WEIGHT VARIATION	PERIOD OF TIME [2]
			inches	*pounds*	*pounds*	
C.S.	Female	34	68½	149	19	Nov. 1939–Feb. 1941
K.J.	Female	24	61	108	5	Nov. 1939–Apr. 1940
H.L.	Male	25	71	164	9	Feb. 1940–Feb. 1941
E.P.	Male	31	66½	116	5	Nov. 1939–Apr. 1940
H.H.	Female	40	62	131	7	Nov. 1939–Feb. 1941
J.S.	Female	22	66½	139	7	Sept. 1940–Feb. 1941

[1] Age at completion of experiment.
[2] The experiments reported in this study come within the periods stated.

after the subject had started to eat his breakfast, but before he had taken the ascorbic acid supplement. Determinations were completed as quickly as possible; the entire time spent from the taking of the first blood sample to the titration of the last sample was about 45 to 60 minutes for five determinations done in duplicate. For 230 plasma ascorbic acid determinations the average difference between duplicates was 0.020 mg. per cent. The differences ranged from 0.000 to 0.042 mg. per cent.

Plan of the experiment

The subjects were prepared by saturation of the tissues with vitamin C during a 3-day pre-experimental period (Belser, Hauck and Storvick, '39). They were considered

saturated if they excreted 200 mg. or more of ascorbic acid in 24 hours.

The experimental periods were 28 days in length. Urinary excretion and fasting plasma ascorbic acid values were determined daily. The various levels of ascorbic acid intake[3] on which the subjects were studied are shown in table 2. The ascorbic acid supplement was taken at breakfast. A test dose of 400 mg. of ascorbic acid, in two 200 mg. doses, one at breakfast and the other at luncheon, was given to each subject on the last day of each experiment. At the close of this 24-hour period, a fasting blood sample was drawn and the ascorbic acid content of the plasma determined to obtain the effect of the test dose on the plasma level of ascorbic acid.

RESULTS AND DISCUSSION

Urinary excretion of ascorbic acid

The results obtained during twenty-one 28-day periods on various levels of ascorbic acid intake are recorded in table 2. The mean excretion of ascorbic acid was generally much higher for the first than for the second to fourth weeks due to the influence of the generous intake of ascorbic acid during the pre-experimental period. Most of the adjustment of excretion to the new level of intake took place within the first 2 or 3 days. However, even after this adjustment, the urinary excretion was by no means constant. In general the variations were greatest on the highest levels of ascorbic acid intake. This may be seen most easily by examining the 25-day means and their calculated standard deviations (table 2). A similar variation was noted by Ralli and associates ('39).

The mean daily urinary ascorbic acid excretions differed for the various subjects. On the 200 mg. level of ascorbic acid intake, the women (C.S., K.J., H.H., and J.S.) showed higher mean urinary excretion values for ascorbic acid, ranging from 154 to 164 mg., than the men (H.L. and E.P.), whose

[3] In all cases, the ascorbic acid intake refers to the amount of synthetic ascorbic acid taken in addition to approximately 10 mg. which was contained in the experimental diet.

TABLE 2

The mean,[1] standard deviation, and range of daily urinary excretions of ascorbic acid and fasting plasma ascorbic acid values with coefficient of correlation,[2] and test dose responses for all subjects on various levels of ascorbic acid intake during 28-day experimental periods.

LEVEL OF ASCORBIC ACID INTAKE	SUBJECT	URINARY EXCRETION OF ASCORBIC ACID PER 24 HOURS			FASTING PLASMA ASCORBIC ACID			COEFFICIENT OF CORRELATION [2]	RESPONSE TO TEST DOSE	
		Mean	Standard deviation	Range	Mean	Standard deviation	Range		Ascorbic acid in urine	Ascorbic acid in plasma
		mg.	mg.	mg.	mg. %	mg. %	mg. %		mg.	mg. %
200	C.S.	154	18	105–195	1.58	0.14	1.33–1.91	+0.170	261	1.53
	K.J.	164	14	130–187	1.27	0.18	0.92–1.62	+0.074	289	1.12
	H.L.	135	14	106–171	1.07	0.13	0.78–1.29	+0.308	261	1.12
	E.P.	130	15	99–162	1.41	0.11	1.23–1.64	—0.105	219	1.60
	H.H.	163	13	138–187	1.07	0.24	0.70–1.57	—0.100	267	1.05
	J.S.	158	14	137–199	1.28	0.10	1.07–1.45	—0.177	290	1.51
150	E.P.	89	23	59–150	1.27	0.16	0.97–1.65	+0.396	221	1.51
125	C.S.	82	10	57– 97	1.74	0.16	1.27–1.99	+0.191 [3]	207	1.95
	H.L.	65	11	40– 89	1.05	0.15	0.69–1.31	—0.174	234	1.31
	H.H.	87	11	70–111	1.05	0.09	0.85–1.24	—0.030 [3]	214	1.27
100	C.S.	59	10	35– 81	1.32	0.15	1.04–1.57	+0.403	250	1.71
	H.L.	47	9	38– 69	1.12	0.09	0.94–1.30	+0.306 [3]	207	1.25
	H.H.	65	12	47–107	0.99	0.10	0.78–1.14	—0.453	220	1.14
90	C.S.	49	20	23–107	1.40	0.13	1.12–1.68	—0.065	208	1.43
	H.H.	58	13	42–110	0.84	0.12	0.61–1.17	+0.226	228	1.02
75	K.J.	45	10	30– 66	1.17	0.10	1.00–1.37	+0.437	248	1.31
	H.L.	36	10	22– 65	0.95	0.12	0.74–1.22	+0.468	76	1.17
65	K.J.	38	11	23– 71	1.02	0.10	0.84–1.32	+0.463	229	1.22
55	C.S.	29	8	19– 52	1.24	0.18	0.92–1.54	+0.604	92	1.45
50	H.H.	26	11	18– 58	0.85	0.10	0.55–1.01	+0.436	121	1.03
	J.S.	26	9	18– 58	1.01	0.12	0.80–1.23	+0.599	123	1.45

[1] Mean values were based on 25 of the 28 days, excluding the first 2 days of adjustment to the new level of ascorbic acid intake, and the last day, which represents the response to the test dose.

[2] Values for the coefficient of correlation between the daily urinary excretion of ascorbic acid and fasting plasma ascorbic acid concentration. The significant values of correlation are 0.396 for the 5% level of significance or odds of 19: 1, and 0.505 for the 1% level or odds of 99: 1. The corresponding values are 0.404 and 0.515 when the data for 24 days are used. (Love, H. H., Application of statistical methods to agricultural research, 1937, p. 491.)

[3] Twenty-four day values were used in the calculation of the value of the coefficient of correlation.

mean urinary excretion values were 135 and 130 mg. Similar variations also occurred on the lower levels of intake although there are fewer data available on such levels. There were less marked variations in the mean ascorbic acid excretions in subjects of the same sex (table 2). Data on more subjects are needed before it can be said that men usually excrete less ascorbic acid, on a given level of intake, than women.

Such variations as were noted apparently were not due to differences in urinary pH, since daily determinations using the LaMotte Universal pH outfit, showed only slight variations among the experimental subjects. The median values for the urinary pH on all 28-day periods were as follows: for C.S., 5.6 and for the other subjects, 5.5.

Fasting plasma ascorbic acid values

In contrast to the urinary excretion of ascorbic acid the mean fasting plasma ascorbic acid values were characteristic for each individual, and did not indicate any consistent differences between the men and women subjects (table 2). It will be noted that the mean plasma values for the six subjects on the 200 mg. level of intake, when saturation was maintained, varied from 1.07 to 1.58 mg. per cent. It is unlikely that these six subjects include the extremes which might be observed in a large group of normal adults. Goldsmith, Ogaard and Gowe ('41) report higher fasting plasma values for three ambulatory patients for whom these values were determined weekly. Figure 1 shows the daily urinary ascorbic acid excretion and fasting plasma ascorbic acid values for subjects C.S. and H.H. on several levels of intake. Of the six subjects studied, these two had the highest and lowest mean plasma values on a given level of intake. The differences in fasting plasma ascorbic acid values on the same levels of intake were marked, although the mean urinary excretions of ascorbic acid were similar.

Whereas, in general, the standard deviations of the mean urinary ascorbic acid excretions are greater for the higher than for the lower levels of intake, the standard deviations of

the mean fasting plasma ascorbic acid values show no such relationship (table 2). Although in general, higher plasma ascorbic acid values were obtained when the subjects were on the higher levels of ascorbic acid intake and lower values when they were on the lower ascorbic acid intakes, the range of values on each level of intake was so wide that in only one case was the difference between mean plasma values for two periods more than twice the standard deviation of this difference. The one case in which the difference between mean

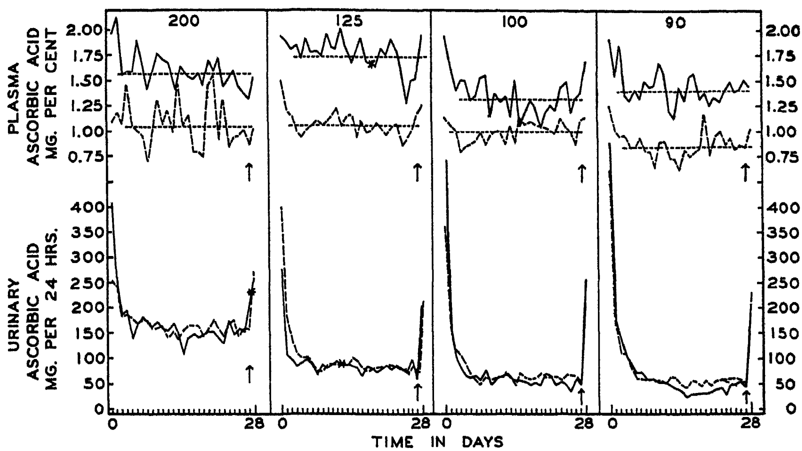

Fig. 1 Daily urinary excretion and fasting plasma ascorbic acid values for two subjects on supplements of 200, 125, 100 and 90 mg. for 28-day periods. Solid lines designate values for subject C.S., dotted lines for subject H.H. Horizontal dotted lines indicate mean fasting plasma ascorbic acid values. An asterisk indicates that a determination is lacking. An arrow indicates a 400 mg. dose of ascorbic acid.

plasma ascorbic acid values on different intakes proved to be statistically significant was when the values for C.S. on the 125 and 55 mg. levels of intake were compared. It is probable that more significant differences would have been observed if the subjects had been studied on lower vitamin C intakes.

The daily variations observed in fasting plasma ascorbic acid values indicate that within the range of intake covered by this study a single determination is of limited value as an index of the state of nutrition with respect to vitamin C. It is true that very high values are likely to indicate adequate

stores of vitamin C and that very low values are usually associated with some degree of depletion of the stores. However, in the present study, a given value for plasma ascorbic acid might be found on widely varying intakes of vitamin C and when the tissue stores were saturated or partially depleted (table 2). For example, values between 1.33 and 1.54 mg. per cent were observed when subject C.S. was receiving supplements of ascorbic acid ranging from 55 to 200 mg. and values between 0.85 and 1.01 were observed when H.H. was receiving ascorbic acid supplements ranging from 50 to 200 mg. This latter subject's mean fasting plasma ascorbic acid value was only 0.84 mg. per cent on a 90 mg. supplement of ascorbic acid, although her output of over 50% of the test dose at the end of the period indicated that she was saturated or nearly so. A similar mean fasting plasma value, 0.85 mg. per cent, was maintained by this subject on a 50 mg. supplement. The response to the test dose following this period indicated partial depletion of the tissue stores (table 2).

As has been suggested by Holmes, Cullen and Nelson ('41), the mean of a series of determinations is probably more useful than individual plasma ascorbic acid values as an index of the state of nutrition with respect to vitamin C. However, a mean plasma value which is indicative of saturation for one subject may signify partial depletion of the stores for another. For example, the highest mean fasting plasma ascorbic acid value exhibited by subject H.H. was 1.07 mg. per cent, when her tissues were saturated on a 200 mg. supplement of ascorbic acid, whereas subject C.S. maintained a mean fasting plasma ascorbic acid value of 1.24 mg. per cent on a daily supplement of 55 mg. of ascorbic acid, which was inadequate to maintain tissue saturation.

Response to test dose and estimation of the amount of ascorbic acid necessary to maintain tissue saturation

The responses to 400 mg. test doses, given at the conclusion of each experimental period, in terms of the urinary excretion of ascorbic acid for 24 hours and the concentration

of ascorbic acid in the plasma on the next morning are shown in table 2.

Although periods of 5 to 7 days may not be sufficient for all subjects to show differences in tissue saturation on low or moderate intakes of ascorbic acid (Belser, Hauck and Storvick, '39), it was believed that they would offer supplementary data on the responses of saturated subjects to the test dose. Data obtained during these short periods are not included in table 2 but each subject was studied during from one to three short periods in addition to a 28-day period on the 200 mg. supplement. This amount of ascorbic acid was considered sufficient to assure tissue saturation.

As has been previously observed (Belser, Hauck and Storvick, '39), considerable variation was noted in responses to the test dose after the several saturation periods. If the lowest response to a test dose after a period on the 200 mg. supplement is used as the criterion of tissue saturation, the following standards are available for these subjects: for C.S., 259; K.J., 249; H.L., 204; E.P., 216; H.H., 267; and J.S., 268 mg. of ascorbic acid. Using these standards, it appears that the following supplements of ascorbic acid were adequate to maintain tissue saturation: for C.S., a little above 100 mg.; K.J., 75 mg.; H.L., 100 mg.; E.P., 150 mg.; and H.H., somewhat above 125 mg. Although E.P. did not receive a supplement less than 150 mg. for a 28-day period, his response to a test dose after receiving 125 mg. of ascorbic acid for 2 weeks was only 187 mg.; hence, he apparently needed more than 125 mg. for tissue saturation. The test dose response given by K.J. after a 2-week period on a 50 mg. supplement of ascorbic acid, was above her standard, but that following the 28-day period on the 65 mg. level of intake was not. There were insufficient data on J.S. to permit the estimation of her requirement for tissue saturation.

The excretion of 50% or more of the test dose within 24 hours has been used commonly as a criterion of saturation (Smith, '38). In this laboratory, no subject has excreted less than 200 mg., that is, 50% of the test dose following a satura-

tion period. If this criterion is used for estimating the smallest supplement of ascorbic acid which maintained tissue saturation for the subjects in this study during the 28-day periods, it appears that the following were sufficient: for C.S., 90 mg.; K.J., 65 mg.; H.L., 100 mg.; E.P., 150 mg.; and H.H., 90 mg. When judged on this basis, test dose responses after 2-week periods, which are not included in the table, indicated tissue saturation for K.J. on a 50 mg. and for H.H. on a 75 mg. supplement, but not for C.S. on a 75 mg. or E.P. on a 125 mg. supplement.

Since all subjects showed a marked urinary response to the test dose on all the levels of intake tested, it may be inferred that none of the levels used was low enough to cause severe depletion of the vitamin C stores during the 28-day period.

In general the fasting plasma ascorbic acid values did not show a rise as a result of the test dose under the conditions of this experiment. In sixteen out of twenty-one cases, the fasting plasma ascorbic acid values following the test dose were within the range for the preceding experimental period. Furthermore, the fasting plasma ascorbic acid values on the morning following the test dose seemed to bear no relationship to the level of ascorbic acid intake for the preceding period. For example, values of 1.45, 1.43, and 1.53 mg. per cent observed in subject C.S. following periods when the supplements were 55, 90, and 200 mg. respectively, are all within the ranges observed on any of the supplements used for this subject.

Whereas Ralli, Friedman and Sherry ('39) concluded that 1.0 mg. per cent represented a normal fasting plasma ascorbic acid value for their subjects during saturation, that an intake of 100 mg. of ascorbic acid was necessary to maintain this level in the plasma, and that higher intakes did not raise the plasma level appreciably, the subjects in this study showed marked individual variation in fasting plasma ascorbic acid values when their tissues were saturated, and they varied markedly in the amounts of ascorbic acid required to maintain this state.

Statistical interpretation of the data [4]

Correlation values [5] were calculated to discover whether or not there was any relationship between the urinary excretion of ascorbic acid and the concentration of ascorbic acid in the plasma when the intake of ascorbic acid was maintained at a constant level. All calculations for the 28-day periods were based on 25-day values which excluded those for the first 2 days of the experimental period, when there was some adjustment to a new level of ascorbic acid intake, and the last day, when the ascorbic acid intake varied from the amount used during the rest of the period. The results are recorded in table 2. For these subjects there was a definite correlation between the daily urinary excretion of ascorbic acid and the concentration of ascorbic acid in the plasma on intakes of 75 mg. and less but no consistently significant correlation between these two variables on higher levels of ascorbic acid intake.

It was also possible to calculate the multiple correlation coefficient when the intake was varied. There were three studies on subject C.S. which were run consecutively. Preceding the 28-day period during which she received 125 mg. of ascorbic acid daily, there were two 14-day periods during which she received no supplement. These periods were separated from each other by several days during which her tissues were resaturated by daily 400 mg. doses of ascorbic acid. Thus there were 65 consecutive days when this subject was on the experimental diet. The value obtained for the multiple correlation coefficient [6] was +0.829 which indicates marked significance, since for the coefficient of correlation for three variables for the degrees of freedom representing 60 days, a value of 0.377 or above is considered highly significant.

[4] The authors are indebted to Dr. H. H. Love and Dr. J. R. Livermore for assistance in the statistical interpretation of the data obtained in these studies.

[5] Croxton, F. E., and Cowden D. J. 1939. Applied general statistics, p. 672. Prentice-Hall Co.

[6] Love, H. H., 1937. Application of statistical methods to agricultural research, pp. 182 and 183. The Commercial Press Limited, Shanghai, China.

Thus there was a high correlation between the urinary excretion of ascorbic acid and the concentration of ascorbic acid in the plasma when there were marked changes in the intake. Inspection of the data indicates that marked increases in urinary excretion of ascorbic acid follow marked increases in intake, but that a similar influence on fasting plasma ascorbic acid occurs only up to the minimum intake required for tissue saturation.

SUMMARY AND CONCLUSIONS

Urinary excretion of ascorbic acid and fasting plasma ascorbic acid values were determined daily in six normal adult subjects, two men and four women, during twenty-one 28-day periods on controlled intakes of ascorbic acid.

In general, lower urinary and plasma ascorbic acid values were observed on the lower levels of intake but there were marked variations in the same subject from day to day, and between individual subjects.

There was a definite correlation between the daily urinary excretion and the plasma concentration of ascorbic acid on the lower levels but not on the higher levels of ascorbic acid intake.

As estimated from the response to the test dose at the conclusion of each experimental period, supplements of from 65 to 150 mg. of ascorbic acid, in addition to approximately 10 mg. in the basal diet, were required by the subjects in this study to maintain tissue saturation.

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NUTRITIONAL STUDIES ON POWDERED WOOL[1]

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THREE FIGURES

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It is generally accepted that keratins such as wool are insoluble in ordinary protein solvents and are not digested by the common proteolytic enzymes. Wool that has been treated with alkali at pH 10-11 (Meunier, Comte and Chambard, '27; Fromageot and Porcherel, '31) or with oxidizing and reducing agents (Linderstrøm-Lang and Duspiva, '35; Stary, '28; Waldschmidt-Leitz and Schuckmann, '29; Goddard and Michaelis, '34) is more susceptible to the action of enzymes.

In preliminary studies (Routh and Lewis, '38) it was observed that after wool was ground in a ball mill the powdered material was readily digested by trypsin and pepsin in vitro. These results suggested the possible utilization of powdered wool by the animal organism. This paper is a report of growth studies on rats in which powdered wool was the main source of dietary nitrogen.

EXPERIMENTAL

The preparation of powdered wool was essentially that described recently in a study of its chemical properties (Routh, '40). Several batches (200–300 gm.) were ground by 500,000 revolutions of the ball mill. The average composition of the

[1] The essential portions of this paper were presented before the thirty-fifth meeting of the American Society of Biological Chemists at Chicago, April 15–19 (1941).

product was 15.70% nitrogen, 3.55% sulfur, 9.03% cystine, and 0.97% ash; the amino nitrogen was 2.23% of the total nitrogen.

Basal diet 15 had the following percentage composition: powdered wool 15, starch 41.4, sucrose 15, agar 2, salt mixture (Hubbell, Mendel and Wakeman, '37) 2.5, hydrogenated cottonseed oil [2] 19, cod liver oil 5, and choline hydrochloride 0.1. Basal diet 20 differed from diet 15 in that it contained 20% powdered wool, 36.4% starch, and 5% cottonseed oil which replaced an equivalent amount of hydrogenated cottonseed oil to overcome the dryness of the diet. As a vitamin supplement each animal received daily 40 µg. riboflavin, 40 µg. thiamine hydrochloride, 200 µg. nicotinic acid and 50 mg. of a rice polish concentrate [3] incorporated in two pills. The pills were fed approximately 12 hours apart. The strain of rats used in this laboratory gained 3–4 gm. per day when 15% casein replaced the powdered wool in the basal diets described above.

Young rats weighing between 37 and 85 gm. were used in these studies. They were housed in individual false-bottomed cages and were fed the basal diets ad libitum. Food consumption and body weights were recorded every 4 days.

Preliminary studies had indicated that animals fed unsupplemented powdered wool as their sole source of protein lost weight less rapidly and lived longer than controls on a nitrogen free diet. To determine which amino acids were needed as supplements to those already present in wool, groups of rats were fed an acid hydrolysate of wool plus various amino acids. In addition to tryptophane which had been destroyed during acid hydrolysis, supplements of both histidine and methionine were found necessary to produce growth. When lysine was also included a slightly better growth was observed.

More extensive studies were then carried out with diets that contained powdered wool supplemented with these amino acids. They were incorporated into the diets at the following

[2] Crisco.

[3] Ryzamin B from Burroughs-Wellcome Co., Tuckahoe, New York; riboflavin and thiamine through the courtesy of Merck and Co.

levels: tryptophane 0.2%, methionine 0.6%, histidine 0.4% (as the monohydrochloride), and lysine 0.6% (as the dihydrochloride). These supplements replaced an equivalent amount of starch in the diet.

Individual growth records are presented in figures 1–3. The curves A to H show the effects of various supplements to the basal diets as outlined in the explanation of the charts.

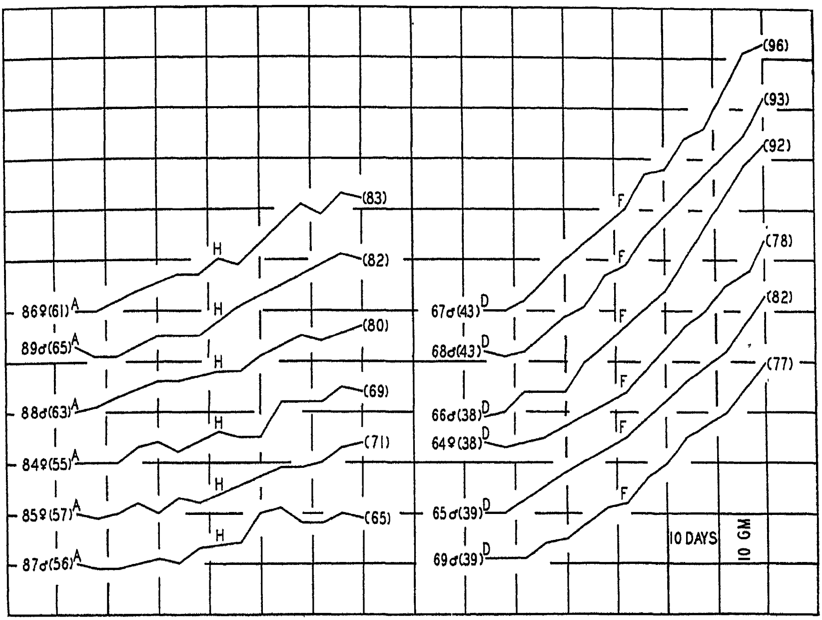

Fig. 1 Initial and final weights are given in parentheses. The various regimes and the food consumption per rat per day were as follows: A — basal diet 15 + 0.6% meth., 0.4% hist., 4.5 gm.; H — basal diet 15 + 0.6% meth., 0.4% hist., 0.6% lysine, 5.0 gm.; D — basal diet 15 + 0.2% trypt., 0.6% meth., 0.4% hist., 4.5 gm.; F — basal diet 20 + 0.2% trypt., 0.6% meth., 0.4% hist., 5.0 gm.

Supplements of tryptophane, methionine and histidine (diet D, figs. 1 and 3) produced moderate growth. When the tryptophane was removed from this diet little more than maintenance resulted (A, fig. 1); addition of lysine (H, fig. 1) did not appreciably increase the rate of growth.

Omission of histidine from diet D caused loss of weight followed by slow growth (B, fig. 2). A somewhat similar

response was observed when methionine was omitted (C, fig. 2).

Growth on diets D and F which contained tryptophane, methionine and histidine was increased by the addition of lysine (E and G). The average gain (fig. 3) in 28 days on diet D was 28 gm., on diet E, 48 gm. The corresponding gain on diet F was 36 gm. and on diet G, 47 gm.

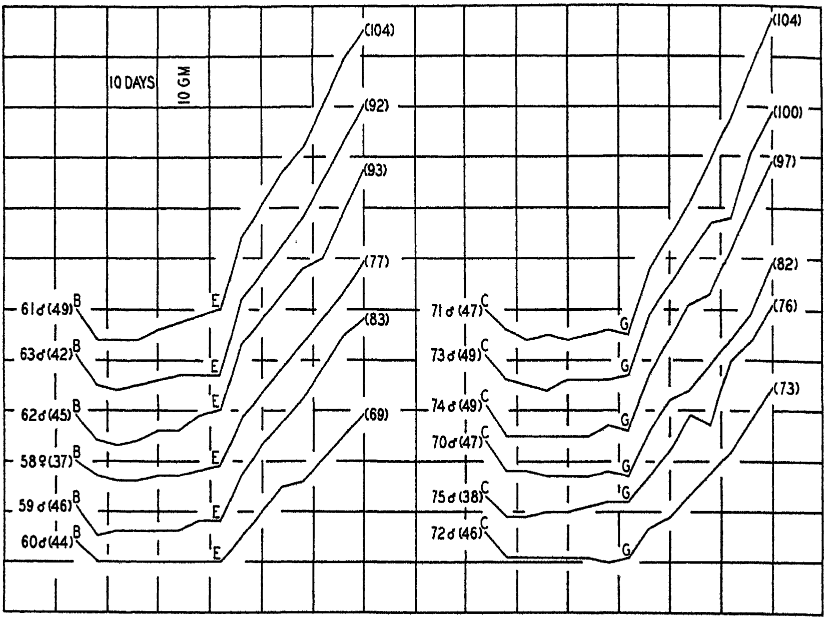

Fig. 2 Initial and final weights are given in parentheses. The various regimes and the food consumption per rat per day were as follows: B — basal diet 15 + 0.2% trypt., 0.6% meth., 2.8 gm.; C — basal diet 15 + 0.2% trypt., 0.4% hist., 2.8 gm.; E — basal diet 15 + 0.2% trypt., 0.6% meth., 0.4% hist., 0.6% lysine, 4.8 gm.; G — basal diet 20 + 0.2% trypt., 0.6% meth., 0.4% hist., 0.6% lysine, 4.7 gm.

A change from basal diet 15 to diet 20 resulted in every case in an increased rate of growth (compare D and F, also E and G, fig. 3). This was probably due to the increased amount of amino acids available.

The average food consumption for the rats on each diet is included in the explanation of the charts. A striking increase

in food consumption was noted when a diet that failed to support growth was replaced by one that produced a moderate growth (compare B and C to E and G). The increase was not as marked when the rats were changed from a diet that produced fair growth to one that resulted in a moderate growth (compare A and D with H and F).

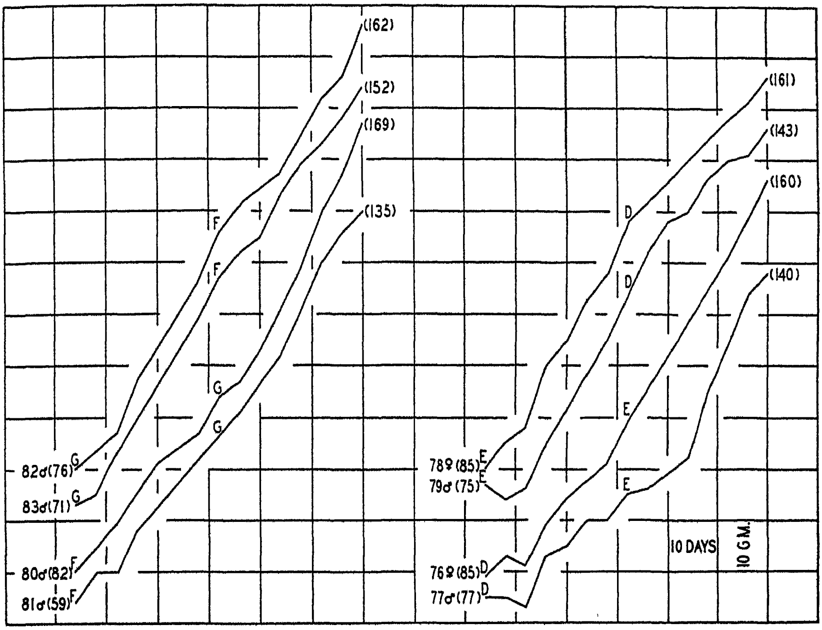

Fig. 3 Initial and final weights are given in parentheses. The various regimes and the food consumption per rat per day were as follows: D — basal diet 15 + 0.2% trypt., 0.6% meth., 0.4% hist., 7.0 gm.; E — basal diet 15 + 0.2% trypt., 0.6% meth., 0.4% hist., 0.6% lysine, 6.7 gm.; F — basal diet 20 + 0.2% trypt., 0.6% meth., 0.4% hist., 6.6 gm.; G — basal diet 20 + 0.2% trypt., 0.6% meth., 0.4% hist., 0.6% lysine, 6.4 gm.

The rate of growth on diet 15 supplemented with tryptophane, methionine, histidine and lysine was slightly greater than that obtained on the wool hydrolysate supplemented with the same amino acids. The average gain on basal diet 15 was 1.5 gm./day as compared to 1.2 gm./day on the wool hydrolysate diet. This suggests that the powdered wool is assimilated quite as readily as the hydrolyzed wool. More

extensive studies on the digestibility and utilization of powdered keratins are being carried out.

CONCLUSIONS

Powdered wool as the sole source of protein in an otherwise adequate diet is incapable of supporting growth in young rats. Tryptophane, methionine, histidine, and lysine were found to be present in suboptimal concentration in the powdered keratin. The supplementation of the basal diet with these amino acids resulted in moderate growth.

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THE INTERRELATIONSHIP OF MANGANESE, PHOSPHATASE AND VITAMIN D IN BONE DEVELOPMENT

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Robison ('23) reported that the phosphatase activity of the bones of rachitic rats is greater than that of the bones of normal rats. Kay ('30) found that an increased phosphatase content of the blood plasma accompanies rickets and certain other generalized bone diseases of human beings such as osteomalacia, osteitis deformans and generalized osteitis fibrosa. Wilkins and Regen ('35) reported that in bone fractures there is a rapid rise in the phosphatase activity at the site of the injury. Auchinachie and Emslie ('33) found that the phosphatase activity of the blood plasma of sheep, fed a rachitogenic diet, is increased three to four times its normal value. On the other hand, Wiese and associates ('39) reported that the activity of the phosphatase of blood plasma and bone is lowered in perosis caused by manganese deficiency. This is a disease of chicks characterized by malformation of the bones. Caskey, Gallup and Norris ('39) presented evidence showing that the ash content of the bones of chicks afflicted with perosis is slightly lower than that of the bones of normal chicks.

Since the phosphatase activity of the blood plasma and bone is greatly increased in rickets and certain other bone diseases of man and other species, and since the activity is markedly reduced in manganese deficiency in chicks, it was thought that, if manganese functions in bone formation by playing an active part in the production of bone phosphatase, this role could

be more clearly demonstrated by observing the effect of manganese deficiency upon bone phosphatase activity and calcification in chicks fed rachitogenic and non-rachitogenic diets. Therefore, studies were conducted in an effort to obtain by this procedure a clearer conception of the function of manganese in bone formation, the results of which are presented in this report.

EXPERIMENTAL PROCEDURE

Two experiments were conducted in this work. Rhode Island Red cockerels were used as the experimental animals. The chicks were divided into groups approximately 24 hours after hatching, weighed individually and confined in metal cages. They were weighed at weekly intervals thereafter and any symptoms of perosis scored as to severity according to the procedure of Wilgus, Norris and Heuser ('37). Feed and water were always available to the chicks. The duration of each experiment was 8 weeks.

The basal diets (A and B) used in these experiments are given in table 1. These rations contained approximately 20% protein. Basal diet A fed in experiment 1 was found by

TABLE 1

Basal diets

INGREDIENTS	DIET A, EXPERIMENT 1	DIET B, EXPERIMENT 2
	%	%
Degerminated yellow corn meal	64.00	64.00
Dried skim milk	10.00	15.00
Meat scrap (55% protein)	10.00	5.00
Dried brewers' yeast	5.00	5.00
Liver meal	5.00	6.50
Soybean oil	2.88	2.88
Calcium carbonate	1.50	1.00
Steamed bone meal	1.00	——
Iodized salt	0.50	0.50
Shark liver oil (2500 I.U. of vitamin A per gram)	0.12	0.12
	100.00	100.00

analysis to contain 2.9 mg. of manganese per 100 gm., and basal diet B fed in experiment 2 was found to contain 2.8 mg. per 100 gm. The amounts of calcium and phosphorus in basal diet A were 1.7% and 0.9% respectively, while those in basal diet B were 1.1% and 0.7% respectively. Both diets produced uniform perosis, but diet B produced the more severe rickets.

Manganous sulfate, c.p., was used as the source of supplementary manganese in these studies and cod liver oil containing 250 I.U. of vitamin D per gram was used to supply supplementary vitamin D.

Experiment 1. In this experiment, two groups of forty chicks each and two groups of twenty chicks each were employed. One group of forty chicks (negative control) received basal diet A only. A second group of twenty chicks received the basal diet plus 5 mg. of manganese per 100 gm. A third group of forty chicks received the basal diet plus 0.5% of cod liver oil. The last group of twenty chicks (positive control) received the basal diet plus 0.5% of cod liver oil and 5 mg. of manganese per 100 gm.

At the end of 4 weeks, eight chicks representative as to body weight and perotic condition were selected from each group and killed, after which the tibiae were removed for analysis. At this time the remaining chicks in the group receiving the diet containing no supplementary manganese and those in the group receiving the diet containing neither supplementary manganese nor vitamin D were each divided into two equal lots. One lot of each group was fed the same diet as previously. The other lot was given its previous diet supplemented with 5 mg. of manganese per 100 gm. At the end of 8 weeks, all the remaining chicks were killed and the tibiae removed for analysis.

The left tibia of each chick was used in determining the moisture, fat and ash content, while the right tibia was used in determining bone phosphatase activity. The right tibia was removed as soon as possible after killing the chick, weighed and cut into small pieces. The pieces of bone were then placed in an Erlenmeyer flask and made to such a volume with dis-

tilled water that the suspension contained 5% of bone. Five drops of chloroform were added, the flask tightly stoppered and the tissue extracted for 60 hours at room temperature. The extract was then filtered and the phosphatase activity of the filtrate determined by the method used by Wiese and associates ('39).

Experiment 2. In this experiment, four groups of twenty-five chicks each were used: One group (negative control) received basal diet B only; a second group received this basal diet plus 7.5 mg. of manganese per 100 gm.; a third received the basal diet plus 0.5% of cod liver oil; the last (positive control) received the basal diet plus 0.5% of cod liver oil and 7.5 mg. of manganese per 100 gm.

Representative chicks were taken from each group at the end of the fourth, sixth and eighth weeks, and analyses were made as in the first experiment. In this experiment, however, the right femur was used for the determination of bone phosphatase activity and the left tibia for the determination of moisture, fat and ash content, while the right tibia was sectioned longitudinally and stained by the silver nitrate procedure in order to determine whether or not rickets was present.

RESULTS AND DISCUSSION

The results for the bone analyses of experiments 1 and 2 are given in tables 2 and 3, respectively. The bone phosphatase activity of the chicks, fed an adequate diet except for a lack of manganese, was markedly reduced as compared to that of chicks fed the same diet supplemented with manganese (positive controls). This was true for all ages of chicks in both experiments. The results confirm those obtained previously by Wiese and associates ('39).

When the chicks received a rachitogenic diet containing an adequate amount of manganese, the bone phosphatase activity was increased to almost twice the amount found in normal chicks (positive controls) of the same age. These results show that in rachitic chicks the bone phosphatase mechanism reacts

in the same manner as that reported for the rat by Robison ('23).

The phosphatase content of the bones, when manganese was omitted from the rachitogenic diet, remained at approximately the normal level instead of increasing to the abnormally high level of the bones of chicks fed a typical rachitogenic diet. The bone phosphatase activity of chicks, which received no supplementary manganese during the first 4 weeks but received

TABLE 2

Results of experiment 1

TREATMENT	CHICKS	WEIGHT	FEED PER CHICK PER PERIOD	RICKETS PRESENT	PEROSIS INDEX	ASH IN FAT-FREE DRY BONE	PHOSPHATASE ACTIVITY
	no.	*gm.*	*gm.*			*%*	*units* [1]
			4 weeks				
—Mn —D	8	226	429	Yes	37.5	36.6	7.19
+Mn —D	8	313	633	Yes	7.3	36.8	15.24
—Mn +D	8	234	402	No	42.8	45.2	5.02
+Mn +D	8	326	593	No	1.2	45.8	7.71
			8 weeks				
—Mn —D	11	376	713	Yes	54.6	36.0	5.82
+Mn —D [2]	11	589	932	Yes	27.8	37.3	10.21
+Mn —D	11	663	1101	Yes	7.1	39.1	12.70
—Mn +D	13	521	1013	No	51.7	43.3	3.14
+Mn +D [2]	14	685	1168	No	14.1	44.8	4.55
+Mn +D	11	868	1391	No	0.0	45.9	5.01

[1] One phosphatase unit is the amount of enzyme which, when allowed to act upon an excess of disodium phenyl phosphate at pH 9.0 for 30 minutes at 37.5° C., will liberate 1 mg. of phenol.

[2] These groups received no manganese during the first 4 weeks but received 5 mg. of manganese per 100 gm. of diet during the last 4 weeks.

manganese at the rate of 5 mg. per 100 gm. of diet for the last 4 weeks, was intermediate. It is evident from these results that manganese is essential for the abnormally great accumulation of phosphatase in the bones of rachitic chicks as well as for normal bone of chicks. Moreover, it appears possible that manganese is also essential for this function in other species of animals, since in "uncomplicated" rickets in chicks the phosphatase content of the bone increases greatly, the same as in the rat.

The difference in the phosphatase level of the bones of groups of chicks fed rachitogenic diets, with and without an adequate amount of manganese, was much greater than that obtained in the bones of groups of chicks fed non-rachitogenic diets, with and without an adequate amount of this element. The greatly increased difference in bone phosphatase level obtained in these studies by the use of rachitogenic diets provides a more specific procedure for determining the effect of manganese upon the level of bone phosphatase than has been used previously.

TABLE 3

Results of experiment 2

TREATMENT	CHICKS	WEIGHT	FEED PER CHICK PER PERIOD	RICKETS PRESENT	PEROSIS INDEX	ASH IN FAT-FREE DRY BONE	PHOSPHATASE ACTIVITY
	no.	*gm.*	*gm.*			%	*units*
			4 weeks				
—Mn —D	6	216	460	Yes	18.4	33.9	7.70
+Mn —D	6	226	450	Yes	0.2	37.8	12.20
—Mn +D	6	251	444	No	27.6	42.4	4.02
+Mn +D	6	262	521	No	0.2	43.6	6.58
			6 weeks				
—Mn —D	8	352	400	Yes	32.3	34.6	7.72
+Mn —D	8	391	451	Yes	1.5	37.1	13.06
—Mn +D	8	421	485	No	31.5	43.9	2.57
+Mn +D	8	500	606	No	0.0	45.5	6.03
			8 weeks				
—Mn —D	9	473	555	Yes	40.7	36.8	8.87
+Mn —D	9	529	542	Yes	0.5	38.3	14.53
—Mn +D	9	603	604	No	27.6	43.4	3.06
+Mn +D	9	751	805	No	0.0	44.8	6.71

In experiment 2 (table 3) the ash content of the bones of chicks fed an adequate diet except for a deficiency of manganese was lower than that of chicks fed the same diet supplemented with 7.5 mg. of manganese per 100 gm. Also, the ash content of the bones of chicks fed a rachitogenic diet deficient in manganese was lower than that of chicks fed the same diet supplemented with 7.5 mg. of manganese per 100 gm. In experiment 1, the same was true at the end of the 8-week period, but

no differences were found at 4 weeks of age. The bone ash reduction was biologically significant and not due to differences in chick weight, as the bone ash of the 8-week-old chicks, fed a low-manganese diet, was less than that of chicks of a younger age and lower weight fed a diet adequate in manganese. These results are in agreement with those previously reported by Caskey, Gallup and Norris ('39) but not with the conclusions of Wiese and associates ('40) who believed that the slightly greater quantity of calcium and phosphorus in various portions of the tibiae of normal chicks as compared to perotic ones was not significant.

The perosis index was observed to be correlated inversely both with the bone ash and the phosphatase activity. It was also found that, after 4 weeks of age when the chicks showed symptoms of rachitic lameness, the perosis indices of the rachitic lots of chicks were somewhat greater than those of the non-rachitic lots. This is believed to be due to the fact that enlargement of the distal ends of the tibiae and the proximal ends of the tarso-metatarsi occurs in rickets as well as in perosis and cannot be differentiated in arriving at the perosis index. If this is true, then the development of rickets slightly retards the onset of perosis, since the perosis indices at the end of the first 4 weeks of the lots of chicks which did not receive either vitamin D or manganese were less than those of the corresponding lots which received vitamin D.

In experiment 1 the fat content of the bones of the chicks receiving an adequate quantity of manganese was greater than that of the bones of chicks fed a diet deficient in manganese. No important differences in fat content were obtained, on the other hand, in experiment 2. Whether or not this discrepancy in results is related to the differences in the calcium and phosphorus content of the basal diets or to some other causes cannot be determined from the evidence available.

Upon examination of the longitudinal sections of the tibiae after staining with silver nitrate, it was observed that the metaphyses of the tibiae of the chicks receiving an adequate diet except for a deficiency of manganese were only approxi-

mately one-half as wide as the metaphyses of the tibiae of the comparable normally fed chicks. Furthermore, the epiphyses of the tibiae of the chicks fed the manganese-deficient diet were very weakly united to the diaphyses. Upon partial dehydration the epiphyses almost invariably separated automatically from the diaphyses in the stained longitudinal sections of the non-rachitic tibiae of chicks fed a low-manganese diet. This did not occur in the normal tibiae. Titus ('32) has reported that the union of the epiphysis and the diaphysis is delayed in chicks afflicted with perosis.

The lowering of the bone phosphatase level in "uncomplicated" manganese deficiency and in manganese deficiency complicated with rickets shows that there is an intimate relationship between the phosphatase activity of the bones and the manganese content of the diet. The results suggest, moreover, that in manganese deficiency the lowering of the phosphatase level retards bone development with the result that the bones are shortened, the ash content of the bones is reduced and the strength of the union between the epiphysis and the diaphysis is greatly reduced. In view of the results of Caskey, Gallup and Norris ('39) it is probable that the deficiency of manganese in the diet causes a disproportionately greater retardation in the development of bones in chicks during growth than of other parts of the body with the result that the bones are not strong enough to support the weight of the body and malformation occurs. That such malformation is not limited only to the chick is suggested by the results of Miller, Keith, McCarty and Thorp ('40) with swine and by some observations of Barnes, Sperling and Maynard ('41) with the rat. Also, a comparison of the symptoms of perosis with those of "slipped epiphyses" in children, recently reviewed by Ghormley and Fairchild ('40), indicates a possible relationship between these bone malformations.

SUMMARY

In rachitic chicks it was found that the bone phosphatase is increased to an abnormally high level the same as in the

rachitic rat. By omitting manganese from the rachitogenic diet, the bone phosphatase was decreased to approximately the normal amount. The difference in the phosphatase levels in the bones of groups of chicks fed rachitogenic diets, with and without an adequate quantity of manganese, was found to be much greater than the difference in the phosphatase levels in the bones of groups of chicks fed a non-rachitogenic diet, with and without an adequate quantity of manganese.

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FETAL RAT PARATHYROIDS AS AFFECTED BY CHANGES IN MATERNAL SERUM CALCIUM AND PHOSPHORUS THROUGH PARATHYROIDECTOMY AND DIETARY CONTROL

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The general question of fetal autonomy under experimental modification of maternal serum components is a nearly virgin field for research. As an extension of our published work on the effect of pregnancy and dietary influence on the size of the parathyroid gland of albino rats we now consider the effect of these factors on the fetuses of the females previously studied. The work is the morphological aspect of a series of experiments done in collaboration with M. Bodansky and V. Duff.[1] The present paper will compare the effect of synthetic diets when supplied to pregnant rats (1) in relation to their parathyroids and those of their progeny, and (2) the effects of the same diets supplied to parathyroidectomized rats and their progeny.

The diets are from the list of Cox and Imboden ('36). Bodansky and Duff ('41 a), who discuss these diets critically, also tabulate the changes in serum calcium and phosphorus and calculate a coefficient of reproductive success. The coefficient compares dietary effects on average pup weights and litter size in normal and parathyroidectomized animals and is useful in establishing the normal diet in our work on gland size.

Adult parathyroids were removed by operation prior to pregnancy or were excised from the larynges of animals sacri-

[1] The colony was reared and chemical determinations were made in the John Sealy Memorial Laboratory, Galveston, Texas.

ficed at the termination of pregnancy. Newborn pups were sorted by weight, sacrificed, the body cavities opened and the entire body then fixed in 10% formalin plus 1% formic acid. Within about 24 hours each larynx was removed and again fixed for several hours before dehydration with dioxan and infiltration with paraffin. The fetal larynx with the thyroid in situ was cut serially. Methods of weighing from paper sections were the same as for adult parathyroids (Sinclair, '41). We tabulate the weights of single parathyroids.

NORMAL DIET AND REFERENCE VALUES OF PARATHYROID WEIGHTS

Diet 7 was found by Cox and Imboden as well as by Bodansky and Duff to be satisfactory for reproduction. We originally considered it an optimum diet, but if enlargement of the parathyroid gland is any indication of dietary deficiency, diet 7 is not adequate during pregnancy. The adult parathyroid on this diet enlarges from 86 μg. in the virgin state to 114 μg. during the first pregnancy, to 208 μg. during the second pregnancy and does not regress later. However, since this ration seems to be excellent in respect to other aspects of reproduction such as litter size and weight we will consider it the normal for comparison.

The normal fetal parathyroid weight in a large series is 7.7 μg. when the pregnant rat is fed diet 7. Meigs et al. ('19) stated that the effects of deficient diets are not apparent until after several litters have been cast. We find no change either in gland weight or in pup size with successive litters. Variation is not excessive. Gland weights in pups of respective body weights averaged as follows: less than 4 gm., 5.8 μg.; 4.1–5.0 gm., 6.0 μg.; 5.1–5.5 gm., 6.6 μg.; 5.6–6.0 gm., 7.7 μg.; 6.1–6.5 gm., 8.5 μg.; 6.6 gm. and over, 9.8 μg. There are no sex differences. Right-left asymmetry is small and favors neither side. The self-regulating mechanism of the fetus is shown to advantage by comparing the effect of diet 7 with 7 v in which 100 to 400 units of viosterol were added daily. We treated the viosterol fed animals as one group. Serum calcium varied both above and below normal. Gland enlargement was kept at 117 μg. instead of the average 141 for diet 7. Fetal para-

TABLE 1

Fetal parathyroid variation with diet and parathyroidectomy.

DIETS			BEFORE PARATHYROIDECTOMY							AFTER PARATHYROIDECTOMY					
No	Ca %	P %	Mater. serum Ca mg.	Mater. serum P mg.	M. para μg.	Fetal wt. gm.	F. para μg.	Stand. dev.	No.	Mater. serum Ca mg.	Mater. serum P mg.	Fetal wt. gm.	F. para μg.	Stand. dev.	No
7	0.49	0.49	9.10	4.67	141	5.45	7.7	2.7	316	4.65	7.81	3.49	8.8	2.8	113
8	0.49	0.735	8.80	6.07	179	4.95	5.6	1.5	13	3.40	8.00	5.58	9.3	—	2
10	0.49	2.450	9.88	4.97	158	4.88	7.0	1.5	11	3.20	5.25	2.26	11.7	3.1	4
12	0.735	0.495	9.36	5.66	small	5.69	8.0	3.0	11	4.50	7.39	5.43	8.0	3.0	17
16	1.125	0.245	11.77	2.50	124	5.24	5.6	1.5	67	11.50	1.90	4.62	6.0	1.7	68
19	1.125	1.225	8.09	5.26	197	5.19	8.8	1.8	84	3.85	8.34	3.50	8.8	1.9	44
26	0.017	0.245	8.18	3.90	265	5.14	7.7	2.4	117	4.06	5.44	5.14	11.0	2.8	41
27	0.122	0.245	9.14	5.13	191	4.95	5.3	1.7	29	4.24	10.76	3.67	10.2	2.7	22
7 v	+ 100 I.U.		9.15	5.13	117	5.4	7.7	1.8	106	3.90	7.01	5.4	9.3	2.0	110
	+ 400 I.U.		8.82	3.14						4.95	6.83				
27 v	+ 100–200 I.U.		9.34	8.16	122	5.2	6.9	1.6	20	5.0	10.71	5.5	9.3	2.4	18
7 al	+ 4% al. acetate					4.5				7.68	4.23	4.6	6.2	2.2	25
11	total no. cases				296				774						464

Diets are from Cox and Imboden ('36). v = 100 to 400 I.U. of viosterol added daily to diets 7 and 27. al = 4% aluminum acetate added to diet 7. Ca and P per 100 cc. for viosterol and aluminum are averages from Bodansky and Duff, yet to be published, while all other serum values are from the work already published. The pups are samples from their stocks and show the same weights. Maternal parathyroid weights are averages from the complete table by Sinclair ('41).

thyroids show no change whatever, growing to the normal 7.7 μg. with less range of variation than on diet 7 unmodified in any way. Body weights were not increased. It seems as if this value may be regarded as a basis for comparison. All fetal calcium storage is calculated from the table of Bodansky and Duff ('41 b), using as the normal their value for storage under diet 7.

EFFECT OF DIET

The high phosphorus of diet 8 prevents calcium utilization. Serum calcium drops and phosphorus rises and the maternal parathyroid enlarges. Fetuses developed under these conditions average 91% of the size on diet 7 but their parathyroid glands weigh 5.6 μg., which is only 73% normal. Calcium storage is 91% normal.

Under diet 10 phosphorus is eliminated at a very high rate as shown by a serum phosphorus lower than for diet 8. Serum calcium is held at a supernormal level and the parathyroid gland is enlarged 12%. Corresponding to the high maternal serum calcium the gland of the fetus is depressed to 7.0 μg. or 91% normal. The fetuses average 89% normal weight and calcium storage is 90% normal.

Diet 16 is rachitogenic because of its low phosphorus content though it contains a great excess of calcium. Cox and Imboden ('36), keeping pregnant rats on various diets, calculated the ash content of young rats at the end of lactation as a percentage obtained between the lowest and highest group values. For diet 7 this was 93.7 and for diet 16 only 59.2. At birth the fetal ash total is the lowest recorded and calcium storage is 69% normal. According to Bogert and Plass ('23) the fetus maintains a calcium level about 16.2% above that of the mother. This depresses the fetal gland but it does not improve calcium storage.

Diet 27 is low in both calcium and phosphorus, yet the serum levels of both are kept nearly normal. The maternal parathyroid is very large (191 μg.); this depresses the fetal parathyroid. Fetuses are 91% of normal size and their parathyroids are 61% of normal. Addition of viosterol to diet

27 keeps the parathyroid weight below that for diets 7 and 27. Fetuses of this group are nearly normal in body weight, their parathyroids 90% of normal weight, and calcium storage is 94% of normal.

Diet 12 causes a slight shift upward in both serum calcium and phosphorus. Our few data show a small parathyroid. Consistent with it the fetuses from these mothers are heavier by 6%, have enlarged glands and a calcium storage supernormal by 18%.

Diet 19 causes a depression in serum calcium, a slight rise in phosphorus and nearly 30% increase in maternal gland size. Maternal needs prevent any excess hormone reaching the fetus. The fetal size is 5% below and the parathyroid gland 15% above normal. Serum calcium below the critical level (a little above 8 mg./100 cc.) is probably responsible for a drop in fetal calcium and is the efficient parathyroid stimulus. Calcium storage is 91% normal.

Maternal serum does not reflect fully the low calcium content of diet 26 because its low phosphorus permits retention. The parathyroid does reflect it in successive increases with each pregnancy: the first carries it from 86 to 142 μg.; the second carries it to 180, the third to 224, the fifth to 460, the sixth to 743 μg. This enormous gland provides no hormone in excess of maternal needs. Reproduction on this diet is difficult but the progeny, both in number and weight, are nearly normal. This normality extends to the fetal parathyroid which weighs 7.7 μg., like those of diets 7 and 7 v. It extends also to successive litters. Calcium storage at birth is 88% normal when diet 26 has been used throughout and 99% if diet 7 is used during the early part of gestation. In pups at the end of lactation Cox and Imboden calculated the coefficient of calcification (ash content) for diets 26 and 7 to be 92.7 and 93.7 respectively. The maintenance of normal weight of body, glands and bone ash in fetus and suckling rat under such an extreme diet shows remarkable utilization.

We assume that the concentrations of serum calcium and phosphorus found in the rats at term were representative of the serum levels during the effective portion of the cycle of

pregnancy. An enlarged maternal parathyroid is correlated with a subnormal parathyroid under diets 8, 10, 27, and 27 v, using the gland sizes under diet 7 for comparison. Diets 8 and 10 produced opposite effects on maternal serum calcium and phosphorus. The presence of excess parathyroid hormone seems to be of greater weight than the level of calcium if the latter does not reach a critical low value a little above 8.0 mg./100 cc. In order to affect the parathyroid the hormone must necessarily pass the placenta. Since the extract of Collip is a colloidal protein it can hardly be identical with the secretion of the parathyroid gland as it is liberated into the blood stream. While there is some conflicting evidence it seems that molecules of that size do not normally pass the placenta. A necessary stimulus to the growth of the parathyroid gland is the complete utilization of its secretion. Free hormone in the fetal circulation depresses the fetal gland just as parathyroid hormone injection shrinks the adult gland.

Normal pregnancy is a greater strain on the homeostatic mechanism for calcium than is indicated by the measure of serum calcium and phosphorus. It may appear in bone ash measurements after successive pregnancies. The parathyroid gland is a more sensitive indicator and its hypertrophy under what is considered a normal good diet shows that the diet of pregnancy must be specially compounded. Phosphorus interferes very much with full utilization of calcium and must be lowered somewhat while calcium content should be raised. Neither should be altered to an extreme degree because phosphorus is as necessary to normal ossification as is calcium. It is recognized that whole populations may adapt themselves to low calcium intake by improved retention (Nicholls and Nimalasuriya, '39). We do not, however, know the full effect of such adaptation. Shelling ('35) pointed out that tetany in the newborn is associated with maternal hypofunction.

EFFECTS OF PARATHYROIDECTOMY

Parathyroidectomy removes the compensating gland factor and permits dietary deficiencies to be fully expressed. Table 1 shows that maternal parathyroidectomy has enlarged the

fetal parathyroid under all but two diets. We note also that the range of absolute increase (52%) or decrease (20%) of fetal parathyroids, like that due to diet alone, is not as great as occurs in maternal parathyroids. This is expected since the mother serves as a buffer between her dietary intake and the homeostatic mechanism of the fetus. This does not prevent a very considerable drop in fetal calcium storage.

Bodansky and Duff ('41 a) show that the coefficient of reproductive success for diet 7 in parathyroidectomy is only 0.733. After parathyroidectomy diet 7 can in no way be considered optimum for reproductive success. Under diet 7 maternal calcium falls far below the critical value and the fetal parathyroid enlarges 14% while body weight drops to 64% of normal. Hoskins and Snyder ('27, '33) found that in dogs an injection of parathyroid hormone directly into the fetus raised calcium level by nearly 50% without in any way altering that of the mother. This is not a simple physical equilibrium between fetal and maternal circulations. The fetus can extract calcium from a maternal source reduced to half its value. Under diet 7 calcium storage per fetus falls to 46% normal.

Shelling ('35) showed that viosterol could produce hypercalcemia even after parathyroidectomy and regardless of diet. If so, under diet 7 v the fetal gland should be nearly normal or reduced. It is enlarged 21% and is larger than on diet 7 alone. The average pup weight, however, was increased 40% at the same time. Bodansky and Duff (unpublished data) show a calcium increase with 100 I.U. of viosterol daily and an unexplained decrease with 400 I.U.

On diet 8 the fetal glands enlarge 65% from a level below the normal for diet 7 to one above that of diet 7 combined with parathyroidectomy. The adequate stimulus is low serum calcium. Fetal calcium storage is 72% normal.

In parathyroidectomized mothers on diet 10 the fetal gland enlarged 67% and fetuses were only 41% normal weight. Fetal calcium storage is 23%, the lowest for any diet used. The number of cases is too few to be decisive but when one

thyroid structure much like that of Graves' disease. Chandler and Pickett ('40) show that parathyroidectomy does not lower oxygen consumption while it lowers serum calcium, and Boelter and Greenberg ('41) found that lowering serum calcium raises the rate of basal metabolism. Canterow, Stewart and Housel ('38) showed that in dogs hyperparathyroidism was associated with thyroid alteration, and Sinclair ('41) found in a cadaver thyroid hyperplasia associated with two parathyroid tumors. Aub and associates ('29) reported that hyperthyroidism increased calcium loss through both the kidneys and intestine. Halstead (1896) found that after nearly complete thyroidectomy in dogs the pups produced hypertrophied thyroid glands. It is not clear why the fetal thyroid should enlarge. Marine ('08) found that hypertrophy could be prevented by giving the mother either iodine or thyroxine. He thought the fetal gland contained no iodine. Hudson ('31) showed that the thyroxine content of the fetal thyroid could be altered by dosing the mother with iodine but while the mother's thyroxine-iodine content varied between 11 and 127 μg. the fetal secretion did not vary outside the limits 13 to 26 μg. per 100 mg. of thyroid tissue. This illustrates the resistance of the fetus to forced changes.

While this series of experiments has yielded results, most of which are consistent, it has also raised new problems by showing the unsuspected cumulative effects of successive pregnancies on the maternal parathyroid with no similar effects on successive litters. It shows a positive correlation of fetal body and gland weights on a normal diet but this correlation does not hold for other diets especially after parathyroidectomy. In the adult there is no such correlation. It has demonstrated that the fetal gland like the rest of the fetal body is resistant to outside influences but is dependent on the maternal organism for its supplies and is capable of giving a differential response to large variations in that supply. The glands function early and effectively. Although Werelius ('13) had suggested that the fetal parathyroid did not function, Sato ('38) demonstrated that an extract from the fetal calf parathyroid is effective in the rabbit.

CONCLUSIONS

1. Fetal rat parathyroids constitute, at birth, 2.8 mg./Kg. of body weight, which is just about double the ratio in adult rats. Diet alone can force variations from a low 2.14 to high 3.4 and maternal parathyroidectomy extends this group variation from 2.6 to 10.34 mg./Kg. The extreme parathyroid weight ratio produced in the maternal rat after six pregnancies was 12 mg./Kg.

2. Fetal parathyroids in rat pups at term are affected by diet through changes in the maternal serum calcium and phosphorus levels and through the stimulus of excessive maternal parathyroid hormone.

3. A diet balanced in calcium and phosphorus content was accepted, because of its high coefficient of reproduction, as an adequate or even optimum diet. It failed to prevent parathyroid enlargement during pregnancy with cumulative effects in a second pregnancy.

4. A diet containing more calcium and less phosphorus was found to suppress both maternal and fetal parathyroids in normal rats but gave results superior to the balanced diet after parathyroidectomy. If extremely unbalanced, fetal calcium storage suffers badly. Aluminum acetate added to a normal diet acted exactly like a hypercalcemic diet.

5. The fetal parathyroids are depressed by an excess of maternal serum calcium or an excess of parathyroid hormone from an enlarged maternal gland.

6. The fetal parathyroids are stimulated by subnormal maternal serum calcium or an excess of serum phosphorus. Both of these conditions are exaggerated in parathyroidectomy.

7. The fetal parathyroid is, at its largest, only about 5% of the adult size and does not protect the mother against the results of parathyroidectomy. The fetal hormone is not lost by dilution but is able to protect fetal serum calcium levels.

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THE NEED FOR PANTOTHENIC ACID AND AN UNIDENTIFIED FACTOR IN REPRODUCTION IN THE DOMESTIC FOWL

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The importance of pantothenic acid in the nutrition of the chick is well established. Ringrose, Norris and Heuser ('31) suggested that vitamin G was a complex consisting of at least two components, one of which was essentially a growth-promoting factor and the other necessary for the prevention of chick dermatosis. The latter component was subsequently termed the chick antidermatosis vitamin or "filtrate factor." The identity of the antidermatosis vitamin and pantothenic acid was established simultaneously by Jukes ('39 a) and Woolley, Waisman and Elvehjem ('39).

Phillips and Engel ('39) studied the histopathology of pantothenic acid deficiency in the chick and found this vitamin to be necessary for the prevention of thymus involution, liver damage, keratitis and dermatitis of the skin and neuropathology of the spinal cord. Snell, Pennington and Williams ('40) showed that the pantothenic acid content of chick tissues is much greater when the chicks are fed an adequate diet than when they are fed a diet deficient in this vitamin. Studies on the quantitative requirement of chicks for pantothenic acid have been made by Jukes ('39 b) and by Bauernfeind, Norris and Heuser ('41).

The role of pantothenic acid in the nutrition of the hen is not as clearly established as it is in the nutrition of the

chick. Lepkovsky, Taylor, Jukes and Almquist ('38) concluded that the antidermatosis vitamin exercised no function in maintaining either hatchability, egg production or character of embryonic mortality under the conditions of their experiments. Bauernfeind and Norris ('39), on the other hand, reported that a heat-labile, non-adsorbable factor in rice bran filtrate, believed to be identical with the antidermatosis vitamin, is required for reproduction in hens fed a so-called heated diet supplemented with heated liver extract. Failure in reproduction occurred, however, unless this diet was supplemented with a heat-labile, adsorbable factor subsequently designated "factor R." The original factor R has since been shown by Schumacher, Heuser and Norris ('40) to consist of at least two factors, termed factors R and S.

The results of Lepkovsky and associates ('38) showed, however, that the amount of the antidermatosis vitamin in eggs is dependent on the amount in the diet of the hens, and is, therefore, important in the production of chicks resistant to dermatosis. Bauernfeind and Norris ('39) also showed that the amount of the vitamin in the egg, as determined by the resistance of newly hatched chicks to the development of dermatosis, is dependent on its concentration in the diet of the hen. Employing synthetic calcium pantothenate, Snell, Aline, Couch and Pearson ('41) demonstrated that the pantothenic acid content of eggs is directly proportional to the amount present in the diet.

More exact studies on the role of pantothenic acid in nutrition were made possible by both the establishment of its chemical structure and its synthesis by Williams and Major ('40), since this brought about the commercial production of this vitamin. It was decided, therefore, to determine whether or not the heat-labile, non-adsorbable factor reported by Bauernfeind and Norris ('39) is identical with pantothenic acid, and also to study further its role in reproduction in hens. The results of this investigation are presented in this report.

EXPERIMENTAL PROCEDURE

Single Comb White Leghorn pullets reared on an adequate diet were used as the experimental animals in this study. They had been laying for several weeks prior to the beginning of the investigation. Five lots of twelve pullets each were selected for uniformity in egg production, body weight and general physical condition. They were placed in pens equipped with wire floors in order to prevent coprophagy and received identical management, other than dietary, throughout the experiment. One male was placed in each pen at the start of the experiment. Subsequently all males were rotated frequently among the different pens in order to eliminate differences in breeding capacity. The hens were weighed at 2-week intervals, and the eggs were incubated weekly. During incubation the eggs were candled on the seventh, fourteenth and eighteenth days in order to determine fertility and embryonic mortality. In cases of doubtful fertility the eggs were broken and examined.

The composition of the basal diet used in this experimental work is given in table 1. The ration was a modification of the

TABLE 1

Basal diet.

INGREDIENT	
	gm.
Heated yellow corn meal	42.5
Heated wheat flour middlings	20.0
Heated wheat bran	20.0
Purified casein	8.0
Soybean oil	2.5
Cod liver oil	0.5
(1500 I.U. vitamin A, 125 I.U. vitamin D)	
Wheat germ oil	0.5
Pulverized limestone	3.5
Steamed bone meal	2.0
Salt	0.5
Riboflavin	0.0275
Thiamine	0.0150
Nicotinic acid	0.0300
plus	
Concentrate of factors R and S equivalent to 5.0% of yeast	

one reported by Bauernfeind and Norris ('39) and contains an adequate amount of all the known vitamins, other than pantothenic acid, needed to support good hatchability. The cereal portion of the diet was heated in an oven for 36 hours at a temperature of 120°C. prior to being mixed with the other ingredients. This treatment has been shown by Kline, Keenan, Elvehjem and Hart ('32) to destroy most of the pantothenic acid. The casein used in the diet was purified in order to free it of pantothenic acid and other water-soluble vitamins. All diets were mixed at intervals of not longer than 2 weeks in order to prevent destruction of any essential nutritive factor by storage.

When the basal diet was fed to chicks, lesions of the dermatosis typical of pantothenic acid deficiency developed. The syndrome was cured by the addition of pantothenic acid to the diet. A microbiological assay of the diet by the procedure of Pennington, Snell and Williams ('40) showed that it contained approximately 225 μg. of pantothenic acid per 100 gm., which is in agreement with previous observations by this laboratory. This is greatly below the requirement of the chick according to Jukes ('39 b) and Bauernfeind, Norris and Heuser ('41).

The basal diet used originally by Bauernfeind and Norris ('39) contained as a source of riboflavin a liver extract which was heated with the cereals. Later work conducted at this laboratory indicated that this extract was essential for reproduction in hens fed the heated diet. Therefore, it was decided to investigate further the possibility that the liver extract used in the diet contained some unrecognized factor which plays an essential role in reproduction. All liver extract [1] used in the experiment was mixed with a small amount of the unheated cereals and heated in an oven for the same length of time as the cereals. After the heat treatment it was finely ground and mixed at the desired level with the other ingredients. By heating in this way there was eliminated the

[1] Wilson's 95% alcohol soluble liver extract paste.

possibility that any markedly beneficial effect from the liver extract was due to additional pantothenic acid.

Factors R and S used in the basal diet were prepared from yeast according to the method described by Schumacher, Heuser and Norris ('40). Factor S was precipitated in alcohol solution at pH 1 and factor R at pH 7. The filtrate was freed of alcohol and concentrated to a suitable volume. The concentrate was used in these studies, since it had not been determined previously whether or not it contained any additional factor, or factors, benefiting reproduction. It is referred to hereafter as the "yeast filtrate." The yeast filtrate was mixed with the cereals and heated in the same manner as the liver extract.

All lots were initially placed on the basal diet for a preliminary period of 8 weeks (period 1) in order to deplete them of their reserve of pantothenic acid. This depletion period was followed by two experimental periods of 10 weeks each (periods 2 and 3) during which time the different lots received pantothenic acid, liver extract and yeast filtrate in various combinations and amounts. The pantothenic acid used in this experimental work was supplied in the form of synthetic calcium pantothenate, dextrorotatory.[2]

RESULTS AND DISCUSSION

The results of the experimental work on reproduction are summarized in table 2. At the end of 4 weeks' depletion, during which all lots of birds received the basal diet, the hatchability was nearly zero in all cases. After 8 weeks' depletion the hatchability of all groups of eggs was zero. The basal diet was markedly deficient, therefore, in a factor, or factors, necessary for reproduction.

During period 2 the hatchability of the eggs from lot 1, receiving the basal ration, as well as those from lot 4, receiving the yeast filtrate and heated liver extract, was zero. This showed that these supplements alone were ineffective in promoting hatchability when added to the basal diet.

[2] Merck.

On the other hand, the hatchability of the eggs from lot 2, receiving pantothenic acid in addition to the heated liver extract, gradually increased until it reached a level of 50–60% which was maintained with some variation until the end of the period. The eggs from lot 5, which received yeast filtrate in

TABLE 2

Per cent hatchability of fertile eggs.[1]

WEEK	LOT 1	LOT 2	LOT 3	LOT 4	LOT 5
			Period 2 [2]		
	Basal	Pantothenic acid + liver extract	Pantothenic acid + yeast filtrate	Liver extract + yeast filtrate	Pantothenic acid + liver extract + yeast filtrate
9	0.0	5.0	0.0	0.0	5.0
10	0.0	31.0	0.0	0.0	0.0
11	0.0	34.8	7.2	0.0	53.6
12	0.0	48.0	27.7	0.0	56.0
13	0.0	55.0	18.2	0.0	53.0
14	0.0	58.0	26.0	0.0	50.0
15	0.0	53.0	27.0	0.0	42.0
16	0.0	59.1	20.0	0.0	47.6
17	0.0	50.0	42.9	0.0	52.2
18	0.0	46.3	34.2	0.0	58.1
12–18 inc.	0.0	52.8	28.0	0.0	51.3
			Period 3 [3]		
	Pantothenic acid	Liver extract	Basal	Pantothenic acid + liver extract	Pantothenic acid
19	2.3	71.0	24.4	19.2	68.8
20	13.3	55.9	18.2	79.1	61.3
21	12.5	50.0	16.3	63.2	60.8
22	12.6	47.2	20.0	57.2	21.9
23	14.3	25.7	0.0	59.3	17.4
24	16.6	19.5	0.0	50.0	38.7
25	12.5	17.1	0.0	67.9	36.8
26	14.2	17.3	0.0	60.7	34.2
27	9.7	0.0	0.0	54.5	35.1
28	13.8	0.0	0.0	65.2	30.5

[1] During the depletion period (period 1) all lots were fed the basal ration. At the end of the eighth week the hatchability of fertile eggs for all lots was zero.

[2] During period 2 the dietary additions per 100 gm. of diet were as follows: 1.0 mg. calcium pantothenate, dextrorotatory; 0.5 gm. liver extract; yeast filtrate equivalent to 5 gm. of yeast.

[3] During period 3 the calcium pantothenate was increased to 2.0 mg. and the liver extract to 2.0 gm. per 100 gm. of diet.

addition to pantothenic acid and liver extract responded in a fashion similar to those from lot 2. It was evident, therefore, that the heated yeast filtrate contained no additional factor influencing hatchability. The hatchability of the eggs from lot 3, which received the pantothenic acid and yeast filtrate, was approximately 25% compared to zero for those from lot 1, but it did not equal that of the eggs from the lots which received pantothenic acid and liver extract. The hatchability of the eggs from lot 5, which received pantothenic acid, liver extract and yeast filtrate, was approximately the same as that of the eggs from lot 2, receiving pantothenic acid and liver extract but no yeast filtrate.

It is evident from these results that pantothenic acid is necessary for reproduction in hens. It is also apparent that except when heated liver extract was included in the diet, pantothenic acid was unable to raise the hatchability above an average of about 25%. Therefore, the liver extract used in this experimental work contained an additional factor, or factors, required for reproduction. No evidence was obtained that the yeast filtrate influenced hatchability favorably.

The experimental setup for period 3 was designed to confirm and supplement the conclusions drawn from the previous period. The level of synthetic calcium pantothenate fed was increased from 1.0 mg. per 100 gm. of diet to 2.0 mg., and the liver extract was increased from 0.5 gm. to 2.0 gm. The use of the yeast filtrate was discontinued in this period, since it was found previously to be ineffective in promoting hatchability when added to the basal diet along with pantothenic acid and liver extract.

The addition of pantothenic acid to the diet of lot 1, which had received the basal diet only for the preceding 18 weeks and which had produced no chicks for the preceding 14 weeks, immediately increased hatchability to 12–15% where it remained for the rest of the period. This increase in hatchability can be attributed only to the addition of pantothenic acid. The hatchability of the eggs from lot 4, now being fed pantothenic acid in addition to the liver extract that it was

already receiving, increased from zero to approximately 80% in 2 weeks. The hatchability of the eggs from this lot subsequently declined slightly but remained in general above that of the eggs from lots 2 and 5 in period 2. It is possible that this was due to the increased levels of the heated liver extract and pantothenic acid supplied in period 3.

When pantothenic acid was removed from the diets of the birds in lots 2 and 3, the hatchability of their eggs dropped to zero. The decline, however, was much slower in the case of the eggs from the lot receiving the 2.0% of liver extract. This may have been due either to the presence of small amounts of undestroyed pantothenic acid in the heated liver extract or to a sparing effect of the factor, or factors, in liver extract upon pantothenic acid.

The hatchability of the eggs of lot 5, which had received liver extract and pantothenic acid in the previous period and had maintained a hatchability of about 50%, declined to approximately 30% in period 3, after removal of liver extract from the diet. Although the hens of both lots 1 and 5 received only pantothenic acid during the third period, the hatchability of the eggs of lot 5 was significantly better than that of lot 2. This is believed to be due to the fact that during the previous period of 10 weeks the birds of lot 5 received liver extract while those of lot 1 had been fed for 18 weeks on the basal diet only.

The results of the third period confirmed those of the second period showing that pantothenic acid is essential for hatchability and that heated liver extract contains another factor, or factors, required for this purpose. The results indicate, moreover, that the basal diet was incomplete even when supplemented with both pantothenic acid and liver extract, as the hatchability of the eggs from a lot of hens maintained under the same conditions as the experimental hens but receiving a normal diet was approximately 72%. This was above the average hatchability for the lots receiving pantothenic acid and liver extract.

The rate of egg production in all lots decreased from approximately 60% to about 40% at the end of the 8 weeks' depletion period. The rate of egg production of lot 1, which received the basal diet for an additional 10 weeks, made only a slight additional decrease during this time. The rate of production of all lots increased when the supplements were added to their diets. The increases, however, were variable and not always consistent. They were most noticeable when both the pantothenic acid and heated liver extract were added to the diets. It appears from these results, therefore, that these factors favorably influence egg production but that the effect is not great under the conditions of the experiment.

After the hens had been fed the depletion diet for about 7 weeks, it was noticed that about 80% of the birds developed a mild form of dermatosis on the feet and lower shanks. The syndrome was similar to that encountered at times in chicks fed a diet deficient in pantothenic acid but in most cases it was not as severe. It was characterized by cracks and fissures on the bottoms of the feet and by peeling of the scales on the lower shanks and tops of the toes which was profuse at times. No dermatosis developed either at the angles of the mandibles or on the margins of the eyelids. A group of pullets kept under identical conditions but receiving a normal diet did not exhibit these lesions. Gradual recovery from the dermatosis was noted when pantothenic acid was added to the diet. Complete recovery resulted in many cases by the end of the experiment. At this time several of the hens which had not yet recovered were placed on a normal diet and given massive oral doses of pantothenic acid. Recovery was more rapid under these conditions.

All of the hens fed the basal diet lost weight appreciably and failed to regain their normal weight even when the diet was supplemented with both pantothenic acid and heated liver extract. This is further evidence that there is still another deficiency in the basal diet used in this experimental work.

The mortality of the hens of lots 2, 3 and 5 was 16.6% in each case and that of lots 1 and 4 was 33.3%. The latter two

lots received no supplementary pantothenic acid for the first 18 weeks of the experiment. The greater portion of the mortality occurred during this time. The lack of this vitamin, therefore, may have been a contributing cause in the higher mortality of these two lots.

SUMMARY

Evidence is presented which shows that pantothenic acid is required for reproduction in hens. It was found that for this function they require also an unidentified, heat stable factor, or factors, present in liver extract. The addition of pantothenic acid alone to the basal diet increased hatchability from zero to 10–30%. When it was added to the basal diet along with the factor, or factors, in liver extract, hatchability was increased to 50–60%. The factor, or factors, in liver extract had little or no influence on hatchability except in the presence of pantothenic acid. A dermatosis was observed on the feet and shanks of hens fed a diet deficient in pantothenic acid.

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THE INACTIVATION OF VITAMIN B_1 IN DIETS CONTAINING WHOLE FISH

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TWO FIGURES

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A nutritional disease of foxes induced by feeding diets that contain uncooked whole fish has been described under the name of "Chastek paralysis" (Green, '36). In 1937 we described the microscopic pathology of this disease, pointed out the relationship to the feeding of whole uncooked fish, and expressed our opinion from evidence at hand that the disease was caused by a vitamin-B_1 deficiency (Green, '37; Green et al., '37).

In 1938 Carlström and Jonsson reported an outbreak of an acute disease of foxes in Sweden characterized by loss of appetite, ataxia, and acetonemia. Twelve foxes died in a period of 2 weeks on a ranch where a diet of fish and cooked cereals was used. A ration containing yeast stopped the outbreak. These authors believed the disease to result from a vitamin-B_1 deficiency but did not recognize fish as a causal factor. Apparently they were dealing with Chastek paralysis.

Ender and Helgebostad ('39) also appear to have recognized Chastek paralysis in foxes, minks, and ferrets on fur farms

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in Norway. They believed the disease to be due to a vitamin-B_1 deficiency and termed the condition beriberi but did not recognize fish as a factor of importance. By feeding silver foxes a diet of casein, cornstarch, vegetable fat, salt mixture, autoclaved yeast, dried whale meat, cod liver oil, vitamin C, and calcium carbonate, they succeeded in producing an illness similar to that seen on fur farms. Lunde ('39) opposed the ideas of Green that fish was a causative factor and that the condition was fundamentally a vitamin-B_1 deficiency. More recently Kringstad and Lunde ('40) have stated that silver foxes given a ration deficient in vitamin B_1 developed anorexia with loss of weight, followed by convulsions 3 or 4 weeks later. Synthetic vitamin B_1 in the amount of 70 international units a day prevented the symptoms. Injections of thiamine were curative.

In 1940 we (Green and Evans) published a summary of our early findings including a list of the species of fish found to cause the disease, and a brief description of the characteristic pathologic changes in the brain of affected foxes. Coombes ('40) has observed Chastek paralysis and accepted our finding that it is related to the feeding of fish. He considers it as a poisoning and not a deficiency disease. In a recent publication we (Green, Carlson and Evans, '41) demonstrated that Chastek paralysis can be prevented by feeding adequate amounts of thiamine hydrochloride in a fish diet, and confirmed our earlier finding that the disease is a B_1 avitaminosis induced by including whole fish of certain species in a fully adequate ration. We have further shown (Evans, Carlson and Green, in press) that characteristic lesions are present in the central nervous system. The lesions have the distribution and the histologic features which in man are considered typical of Wernicke's hemorrhagic polioencephalitis. In the present paper are reported experiments (1) that demonstrate quantitatively the destruction of thiamine by whole fish and (2) that yield information on the anatomical distribution in the fish of the thiamine-inactivating factor.

Quantitative tests of the amount of thiamine inactivated by a known amount of fish

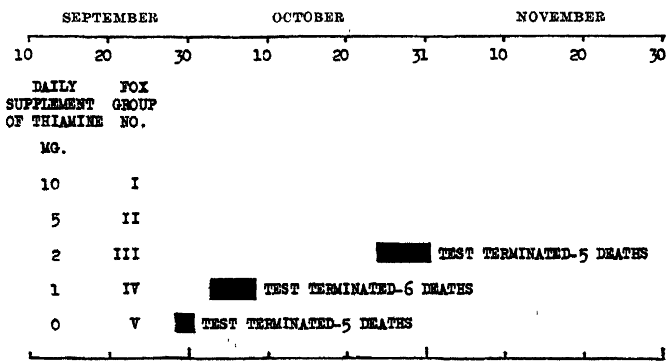
In order to determine approximately the amount of thiamine required to prevent the occurrence of Chastek paralysis in foxes given a ration containing a known amount of fish, we have performed an experiment utilizing fifty red fox pups about 6 months old. The percentage composition of the basal diet in this experiment was the same for all animals; namely, cereal 15, bread 10, carrots 9.5, horse meat 45, whole carp 20, cod liver oil 0.5. The cereal was a mixture of wheat, corn, oats and rice. All ingredients were ground together to make a mixture the consistency of hamburger. Each fox received 12 ounces of feed a day. Thiamine hydrochloride in specified amounts was added as an aqueous solution and thoroughly mixed with all other ingredients of the ration.

The foxes were divided into five groups of ten animals each. Four of the groups were fed daily rations containing 10, 5, 2, and 1 mg. of thiamine hydrochloride, respectively. Foxes in the fifth group received the basal ration without a supplement of thiamine. The experiment was begun on September 4, 1940, and was terminated on December 5. If foxes with severe symptoms did not die quickly, they were etherized. As soon as half the animals in any group had died of Chastek paralysis or had been killed in extremis, the surviving foxes in that group were taken off the experiment. The results obtained in the experiment are shown in figure 1.

We had previously found that 25 mg. of thiamine a day would protect foxes from Chastek paralysis while on a diet containing 20% whole uncooked carp. In the present experiment 10 mg. of thiamine likewise afforded complete protection against the disease. The inclusion of 5 mg. of thiamine in the diet offered complete protection from death and from any neurologic symptoms whatsoever. However, after 7 weeks some animals went off feed at irregular intervals. Loss of appetite is characteristically the first evidence of Chastek paralysis, and it is our opinion that the inappetence presented

by the foxes in group II, fed 5 mg. of thiamine, is a manifestation of subclinical Chastek paralysis. Among the foxes that received 2 mg. of thiamine, inappetence and subsequent fatalities appeared a month later than in the controls. The experiment was terminated for group III after five deaths occurred, and there is no doubt that all animals would have succumbed had the experimental diet been continued. Some slight beneficial effect was produced in group IV by adding 1 mg. of thiamine,

EFFECT OF THIAMINE HYDROCHLORIDE IN THE PREVENTION OF CHASTEX PARALYSIS AMONG FOXES RECEIVING GROUND, WHOLE, RAW CARP AS 20% OF THEIR DIET



Experiment was begun on September 4, 1940.
Period during which deaths occurred.
Test for groups I and II was terminated on December 5, 1940.

Figure 1

inasmuch as Chastek paralysis appeared a week later in that group than in the control foxes, which received no supplement of thiamine. Although symptoms and fatalities were delayed in the groups given 1 mg. and 2 mg. of thiamine, the disease, when it did appear, was as acute and as typical pathologically as it was in the controls.

As each fox received approximately 2.4 ounces of whole carp daily in the one meal offered, we can determine roughly the amount of thiamine inactivated by a pound of carp and the interrelationship between the degree of inactivation and the

disease produced at the different levels of thiamine fed (table 1). We have no accurate knowledge of the thiamine content of the basal diet; but if frozen horse meat has approximately the same thiamine content as lean beef, there were probably about 50 I.U. of thiamine in each 12-ounce feeding. Our estimates of the amount of thiamine inactivated by a pound of carp are conservative, since they take into account only the thiamine added as a supplement to the ration.

TABLE 1

The inactivating effect of one pound of carp on different amounts of thiamine.

FOX GROUP NO.	DAILY THIAMINE SUPPLEMENT	AMOUNT OF THIAMINE TO EACH POUND OF CARP [1]		EFFECT ON FOXES
		Weight	International units	
	mg.	*mg.*		
I	10	67.0	22,000	No deaths; no symptoms in 92 days.
II	5	33.5	11,000	No deaths; mild symptoms a month after controls died.
III	2	13.4	4,400	Deaths delayed 1 month.
IV	1	6.7	2,200	Deaths delayed 1 week.

[1] Figures indicate the amount of thiamine added to 5 pounds of ration (each 5 pounds of ration contained 1 pound of carp). The relatively small amount of thiamine occurring naturally in the basal diet (see text) is not included in these figures.

From the data shown in table 1 it is obvious that when fish is fed as 20% of the ration, 1 pound of carp will inactivate supplemental thiamine in excess of 4,400 units; but 1 pound of carp will not completely inactivate 22,000 units.

The actual amount of thiamine that a pound of carp inactivates is not a constant value but is variable, depending upon the amount of thiamine mixed with the fish. One pound of carp apparently failed to inactivate completely 2,200 units of thiamine in the case of group IV, yet a like amount of carp in group II must have inactivated about 10,000 units. This dependence of the amount of thiamine inactivated upon the

amount of thiamine available suggests adsorption as the basis of the inactivation, rather than a simple chemical reaction between two substances. It should be emphasized that the term inactivation is used only to describe the gross result of feeding carp and thiamine in the same ration. The actual physical, chemical, and physiologic phenomena concerned are still a matter for conjecture.

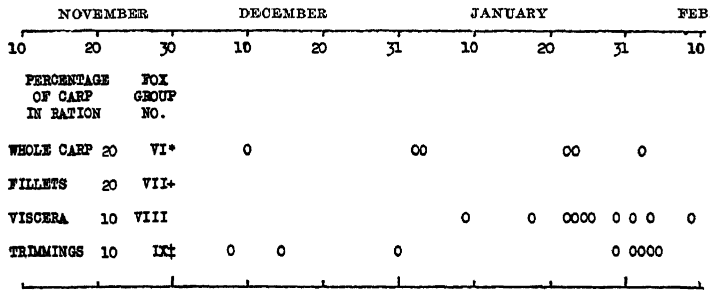
Study of the potency of various organs of fish in producing Chastek paralysis

In order to determine which organs of fish contain the substance that causes Chastek paralysis, an experiment in which forty red foxes were used, was begun in November, 1940. The carp fed in this experiment were freshly seined. Some were used as whole fish; others were eviscerated and skinned, after which the muscle was removed from the skeleton as fillets. The gastro-intestinal tract, liver and other viscera were frozen in one container; the fillets, in a second; and the heads, fins, tails, and skin, with one-fourth of the original amount of scales, were frozen in a third container. The basal diet in this experiment had the following percentage composition: cereal 15, bread 10, carrots 9.5, cod liver oil 0.5, horse meat and fish 65. Through an error, fresh unfrozen horse meat was used instead of frozen horse meat during the first month of this experiment. On December 13 fresh meat was discontinued and the frozen product was used thereafter. In figure 2 are listed the fish ingredients of the diet given each group of ten foxes and the results obtained. All foxes that died displayed typical symptoms of Chastek paralysis. The experiment was begun on November 13, 1940, and terminated on February 26, 1941, when all the foxes fed viscera and all those fed trimmings (heads, skin, fins and skeleton) had succumbed to the disease.

It is clear from the results that the agent in whole carp which is responsible for Chastek paralysis is found in the external structures of the fish and in the viscera, but is not present to any comparable degree in the somatic muscle. It was also apparent during the course of the experiment that

the carp viscera and trimmings were fully as effective as whole fish, or more effective than whole fish, in the production of Chastek paralysis. At the time the experiment was terminated, only six of the ten foxes that received whole fish had succumbed, while the foxes that received viscera and trimmings had all died. Three cases of Chastek paralysis developed while the foxes were receiving fresh, unfrozen horse meat. The greatest grouping of deaths came around January 25, 2½

POTENCY OF DIFFERENT ANATOMIC FRACTIONS OF CARP IN PRODUCING CHASTEX PARALYSIS



Experiment was begun on November 13, 1940, and terminated on February 26, 1941.
Fresh, unfrozen horse meat was used until December 13; frozen horse meat was used after that date.
0 - Death of a fox.
* Six of the 10 animals in this group died of Chastek paralysis or were killed in extremis. The remaining 4 foxes were injected with thiamine on February 26 and placed on a diet that contained liver but no fish.
\+ Foxes receiving the carp fillets remained entirely well.
‡ Two remaining foxes in this group died on February 26, 1941.

Figure 2

months after fish was added to the ration and 6 weeks after the substitution of frozen horse meat for the fresh product. The quick and decisive development of Chastek paralysis by the foxes that received whole fish in the first experiment (fig. 1) was not at all duplicated by the foxes in the second experiment (fig. 2) that were fed whole fish. The experimental details were identical for the two groups except for the period in which fresh horse meat was fed to the latter group of foxes.

DISCUSSION

From our series of experiments on Chastek paralysis, it seems established that the disease is not analogous to beri-

beri, which is known to result from a deficiency of several vitamins, but rather to Wernicke's disease in which there is probably a deficiency of only vitamin B_1 (Alexander, '40). The regularity with which we have been able to prevent the development of Chastek paralysis by the use of crystalline thiamine indicates that in Chastek paralysis the deficiency concerns only this one substance, and that other vitamins are present in an adequate amount.

Recently, György, Rose, Eakin, Snell and Williams ('41) have presented experimental evidence that "egg-white injury" results from the inactivation of biotin by a component of egg white which they call "avidalbumen". The mechanism they describe closely parallels that which we postulated as operative in Chastek paralysis. The similarity extends even to the fact that the noxious substance in egg white, like that in fish, is destroyed by cooking. Complete proof has not yet been obtained, but the most probable basis for each disease appears to be the inactivation of a specific vitamin by a constituent of the diet.

Beck and Peacock ('41) have carried out dietary investigations in relation to papillomatosis of the fore-stomach of the rat and have concluded that "the mechanism of papillomatosis in these experiments seems to be related to an induced avitaminosis-A due to the presence in repeatedly heated fats of an 'anti-vitamin A' of undetermined nature".

Mackenzie, Mackenzie and McCollum ('41) have shown that the muscular dystrophy induced by feeding cod liver oil to herbivores (rabbits, in their experiments) can be prevented by giving the animals very large doses of alpha-tocopherol.

Apparently the adequacy of a given amount of vitamins A and E, as well as of biotin and thiamine, can be unfavorably affected by other ingredients of the diet. The possibility that a dietary constituent may destroy a given vitamin or in some other way cause a normally adequate supply of that vitamin to become insufficient may be a factor of greater importance in human and veterinary medicine than has been generally recognized.

Certain fur farmers have fed whole carp without untoward results by giving the animals chunks of carp 1 day, and giving them a balanced ration without whole fish the following day — alternating the fish and other feed in a systematic manner. In fact, some fur farmers have used whole carp with evident success by giving the fish alone in chunks in the morning and giving a balanced ration as a second or evening meal. On ranches on which fish has been mixed with the total diet but fed only 3 or 4 days a week, no difficulty with Chastek paralysis seems to have been encountered. Although it takes very large amounts of thiamine mixed with fish to prevent Chastek paralysis, a relatively small amount of vitamin B_1 consumed at a time when it will not be mixed with the fish either in the feed pan or in the gastro-intestinal tract is evidently sufficient to prevent the development of Chastek paralysis. This point is of considerable practical importance. It has not yet been experimentally tested but seems fairly well established on the basis of ranch observations.

SUMMARY

1. Among foxes fed a ration containing 20% whole carp and variable amounts of thiamine, Chastek paralysis failed to develop in those given 10 mg. of thiamine each day, and manifested itself only as transient anorexia among those given 5 mg. of thiamine. The onset of Chastek paralysis was delayed 1 month among foxes given 2 mg. of thiamine hydrochloride each day, and then appeared in this group in the usual fatal form.

2. A group of foxes fed skin, scales, fins, skeletons and heads of carp developed Chastek paralysis; another group fed the carp viscera contracted the disease; those given muscle as carp fillets showed no evidence of Chastek paralysis.

3. Observation on fur ranches where fish has been used indicates that Chastek paralysis will not develop if whole fish is fed several days a week and a diet containing adequate amounts of vitamin B_1 is fed on the other days of the week.

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BIOTIN IN CHICK NUTRITION [1]

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In a previous paper (Hegsted, Oleson, Mills, Elvehjem and Hart, '40) we described a typical scaly dermatitis in chicks fed purified rations, which condition was not prevented by pantothenic acid. Distribution and properties of the protective factor agreed with those reported for vitamin H, and potent preparations of vitamin H produced cures of the dermatitis. György, Melville, Burk and du Vigneaud ('40) first noted the possible identity of biotin and vitamin H. A short while later du Vigneaud, Melville, György and Rose ('40) demonstrated the vitamin H activity of crystalline biotin methyl ester in rats showing egg white injury. Purification of du Vigneaud's crystalline biotin methyl ester to constant melting point and constant biotin activity produced crystals of such potent vitamin H activity that there was no longer any doubt that biotin and vitamin H were the same compound (György, Rose, Hofmann, Melville and du Vigneaud, '40). Various biotin preparations have subsequently been used in studies of the dermatitis. Ansbacher and Landy ('41) found that crystalline biotin methyl ester completely cured a severe dermatosis similar to that described by us, but produced on a heated ration.

We wish to present here further studies on the dermatitis as produced on purified rations. Both biotin concentrates and

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the Wisconsin Alumni Research Foundation and the Works Progress Administration.

free crystalline biotin have prevented the condition and promoted growth in chicks.

EXPERIMENTAL

The basal rations used have had the following percentage composition: dextrin 55, purified casein 18, salts IV (Hegsted, Mills, Elvehjem and Hart, '41) 5, defatted cartilage 15, soybean oil 5, and solubilized liver extract 2. Each kilo of ration contained inositol 1 gm., thiamine 3 mg., pyridoxine and riboflavin 4 mg. each, calcium pantothenate 15 mg., nicotinic acid 100 mg. and choline chloride 1.5 gm.[2] In certain of the groups receiving molasses the solubilized liver extract was omitted and in others a concentrate prepared from solubilized liver extract (Hutchings, Bohonos, Hegsted, Elvehjem and Peterson, '41) was substituted for it. Each chick received 2 drops of fortified haliver oil per week, supplying adequate amounts of vitamins A and D.

Day-old White Leghorn chicks were used. They were kept on raised screens in small heated brooders and given the experimental ration and water ad libitum.

RESULTS

As previously reported, lesions started to appear at 2–3 weeks and were quite severe at 4 weeks. The syndrome is characterized by lesions first appearing on the bottoms of the feet, followed by mandibular lesions. About 80% of all the chicks on the basal ration showed the dermatitis at 4 weeks.

Table 1 shows the effectiveness of various supplements in preventing the dermatitis. Molasses, a fair source of biotin, prevented it almost completely at a level of 10% of the ration. Biotin concentrates[3] also were effective. A level of 5 μg. of

[2] We are indebted to Merck and Co., Inc., Rahway, N. J., for the thiamine, pyridoxine, riboflavin, calcium pantothenate, nicotinic acid and choline chloride; to the Wilson Laboratories, Chicago, Ill., for the solubilized liver extract and cartilage; to Allied Mills, Peoria, Ill., for the soybean oil; and to Abbott Laboratories, North Chicago, Ill., for the haliver oil.

[3] Generously supplied by Dr. Maurice Landy of the Research Laboratories, S.M.A. Corporation, Chagrin Falls, Ohio.

biotin in the form of this concentrate per 100 gm. of the ration was only partially effective — of the six chicks, three had slight lesions. Levels of 10 and 20 μg. per 100 gm. completely prevented the dermatitis. One group of chicks was given free crystalline biotin (du Vigneaud)[4] at a level of 10 μg. per 100 gm. of ration. In this group one chick showed slight lesions at 3 weeks, which were cured within a week by two injections of 2.5 μg. of the crystalline biotin. The other chicks were completely protected.

TABLE 1

Effect of supplements on the dermatitis in chicks.

SUPPLEMENT TO BASAL RATION	NUMBER OF CHICKS	PER CENT WITH DERMATITIS
None	95	80
10% molasses	54	4
5 μg. biotin (S.M.A.) per 100 gm.	6	50
10 μg. biotin (S.M.A.) per 100 gm.	6	0
10 μg. biotin (crystalline) per 100 gm.	3	0
20 μg. biotin (S.M.A.) per 100 gm.	17	0
30 mg. para-aminobenzoic acid per 100 gm.	5	100

In addition to preventing the dermatitis, biotin is also a growth factor for chicks. Those on the basal ration weighed an average of 140 gm. at 4 weeks, while those receiving 20 μg. of biotin per 100 gm. weighed 193 gm. Chicks getting 10 μg. of crystalline biotin weighed 182 gm.

Para-aminobenzoic acid neither prevented the dermatitis nor promoted growth on this ration.

Data on the biotin content of the livers and kidneys of some of the chicks showing dermatitis, and some receiving various levels of biotin are presented in table 2. Stores of biotin in the liver and kidney are very low in the chicks having dermatitis, while the biotin content of the organs of the positive controls is almost four times as great per gram. On the basis of total biotin per organ the differences are still greater because of the difference in the size of the chicks.

[4] We wish to express our appreciation to Doctor du Vigneaud, Cornell Medical School, New York, for the sample of free crystalline biotin.

TABLE 2

Biotin content of chick tissues.[1]

SUPPLEMENT TO BASAL RATION	NUMBER OF CHICKS	NUMBER WITH DERMATITIS	BIOTIN CONTENT OF TISSUES-DRY	
			Liver	Kidney
			µg./gm.	*µg./gm.*
None	10	10	3.3	1.3
10 µg. biotin (crystalline) per 100 gm.	3	0	8.2	2.5
20 µg. biotin (S.M.A.) per 100 gm.	5	0	12.0	6.0
3% kidney residue	6	0	11.9	20.0

[1] Analyses were by the methods of Snell, Eakin and Williams ('40) and Lampen, Kline and Peterson ('41). We wish to thank Miss Margaret McGregor and Mr. J. O. Lampen for making these analyses.

DISCUSSION

The prevention and cure of the typical dermatitis by crystalline biotin and biotin concentrates confirm our earlier observations that the dermatitis is the result of a biotin deficiency. The growth stimulating properties of biotin place it in the already long list of essential growth factors for the chick.

The biotin requirement of the chick can be roughly estimated from our data. In addition to that present in the basal ration, the minimum amount required to prevent dermatitis and give maximum growth seems to be about 10 µg. per 100 gm. of ration. Five microgram per 100 gm. is inadequate, as can be seen from the 50% incidence of the dermatitis. The one slight case at 10 µg. may indicate that this level is marginal. Molasses contains 0.7 µg. per gram so that 10% of molasses supplies 7 µg. per 100 gm., slightly below the level given above as marginal. Three per cent of kidney residue [5] in the ration has been found adequate to prevent dermatitis in thousands of chicks in other studies here. The biotin content of the kidney residue is about 2.5 µg. per gram, so that 7.5 µg.

[5] The residue after exhaustive extraction of defatted kidney with water, 50% ethanol, and 50% methanol.

per 100 gm. is supplied by it. From all these results, we can conclude that chicks need 7–10 μg. of biotin per 100 gm. of ration in addition to the amount found in our basal ration.

SUMMARY

1. Biotin concentrates and free crystalline biotin have been found to prevent a typical dermatitis and to promote growth in chicks.

2. The biotin requirement of the chick is at least 7–10 μg. per 100 gm. of ration.

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THE IRON REQUIREMENT OF CHILDREN OF THE EARLY SCHOOL AGE

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Although a number of studies have been made on the iron requirement of infants and preschool children, little is known concerning the requirement of the child of school age. It is the purpose of this study to make a contribution toward filling in this gap.

The method which has been used previously to determine the iron requirement has been that of the balance study. This method is a suitable one for finding the requirement of adults because the object is to find the lowest level at which the intake and outgo exactly balance. It is not necessarily applicable in the case of children, where the purpose has been to find an intake giving a large or even a maximum retention. It has been assumed that any excess beyond that needed to replace catabolic losses, and to furnish iron for growth as well as for a reasonable storage, would be excreted: that is, a child would not retain more iron than he needed. Of recent years experimental work has been done which indicates that this assumption was false.

Evidence that excess iron is not excreted was furnished by McCance and Widdowson ('38) who found that intravenous injections of iron in subjects on a constant iron intake resulted in practically no increase in fecal iron, and also by Hahn et al. ('39) who injected radio active iron in dogs and found that practically all the injected iron was retained, even though the dogs were so well stocked with iron that there was obviously no need for retention.

If excess iron is not excreted, large amounts will accumulate on high intakes. Such has been found to be the case by Hutchison ('37) who reported that when children were given large amounts of iron orally, all had large retentions as shown by balance experiments. The retention of one child in only 3.5 months was 3.5 gm., which is more than he could possibly have needed. Adults, too, have been shown to retain correspondingly large amounts on high iron intakes.

Since excess iron is not excreted, retentions may be much greater than the amount needed and there seems to be no way to tell how large retention should be. This being the case, it is necessary to find some criterion other than the size of the retention on a given intake for determining the amount of iron needed. Because a large proportion of the iron in the body is found in the hemoglobin, and because this is probably one of the first iron-containing tissues to be depleted when there is a deficiency, it would seem that a good hemoglobin level could be taken as an indication of an adequate iron supply. If so, the procedure would be to find the lowest level of iron intake that will produce and maintain a good hemoglobin level.

EXPERIMENTAL

The general plan. Subjects were selected who had been living for several years in an institution where the diet was fairly uniform, in order that their hemoglobin levels could be considered a product of that diet. Their hemoglobin levels were determined and iron analyses were made on their diets. They were then matched in groups of three and given graded amounts of iron. After the iron supplementation had been continued for 7 months, hemoglobin levels were again determined and the data examined to find the lowest intake that gave good hemoglobin levels.

The subjects. This study was carried out at Mooseheart, an institution whose standards of nutrition and physical care are high. Twelve boys and nine girls, from the ages 8 to 11 years were selected as subjects. As a group, their nutritional condition rated higher than average. None of them had had

iron therapy or had suffered severe or chronic blood loss. All but one had been living on the diet of the institution for 2 years or more. It seemed, therefore, that their hemoglobins would be the product of that diet.

The diet. The diet was a good one containing all the foods customarily advised for children. Calculations made on the diets of the subjects showed that all were up to or above the daily allowances recommended by the Committee on Food and Nutrition of the National Research Council ('41) for every dietary essential. Probably, therefore, the diet furnished all the dietary factors which affect hemoglobin.

The blood study. Capillary blood was taken from the ear lobe. Hemoglobin determinations were made in duplicate by the method of Evelyn ('36) using the Evelyn Photoelectric Colorimeter. The red cells were counted from two pipettes of blood on a Bright-line Improved Neubauer Haemacytometer. The blood samples were taken during the time the dietary study was being made, on two different days, a week or two apart, always at the same time of day for the same child. The children were kept sitting quietly for at least 45 minutes before each test, and were kept as free as possible from excitement.

The individual hemoglobin values ranged from 13.8 to 11.6 gm. per 100 ml. of blood (table 1). The mean hemoglobin for all twenty-one children was 12.9 gm. The red cell counts ranged from 3,810,000 to 4,730,000 cells per cubic millimeter with a mean of 4,160,000 (table 2). There was no significant difference between the two sexes; the mean for the boys was 12.7 gm. of hemoglobin and 4,200,000 red blood cells per cubic millimeter and for the girls was 13 gm. and 4,120,000 cells. Since the analytical method was not accurate to more than 0.5 gm., all those hemoglobins falling between 12.5 and 14.5 gm. were within experimental error of the zone 13 to 14 gm., an area which is generally accepted as high normal.[1] Sixteen

[1] Evidence that the value 13–14 gm. of hemoglobin is a normal level for children in excellent health was obtained by an examination of the hemoglobin values of the state winners of the 4-H Club health contests. This information was made available by the Elizabeth McCormick Memorial Fund.

of the children fell in this zone; three fell in the range of 12 to 12.5 gm., which would still be considered normal; two fell in the range of 11 to 12 gm. which might be called low normal; and none even approached an anemic level which is usually considered as 10 gm. or below.

The iron intake of the subjects. The dietary study extended over a period of 2 weeks. A period of this length was used because it had been found by previous studies, made in this laboratory (McClelland, '31), that this is the shortest period

TABLE 1

The relationship of the average daily iron intake to the original hemoglobin level.

SUBJECT AND SEX	AGE	HEIGHT	WEIGHT	HEMOGLOBIN	TOTAL IRON	IRON PER KILOGRAM
	yr.–mo.	*cm.*	*kg.*	*gm.*	*mg.*	*mg.*
T.R.—M	9– 5	137.3	29.4	13.8	14.6	0.50
C.W.—F	10–10	143.4	33.4	13.6	11.4	0.35
B.H.—M	9– 0	131.8	32.2	13.5	14.8	0.46
B.C.—M	10– 0	137.0	32.6	13.4	12.5	0.38
D.S.—F	10– 4	140.3	33.4	13.3	11.6	0.35
G.Ho.—F	10– 9	141.5	37.7	13.3	11.7	0.31
B.Co.—F	10– 1	135.0	31.6	13.2	11.9	0.38
R.C.—F	9– 2	136.8	31.2	13.1	10.8	0.35
H.S.—F	10– 5	135.6	39.0	13.1	12.0	0.31
J.J.—M	8– 8	130.4	28.6	13.0	12.8	0.45
B.W.—F	9– 4	142.3	31.7	13.0	11.7	0.37
A.H.—F	11– 3	147.5	43.3	13.0	13.0	0.30
G.H.—M	8– 3	130.9	28.2	12.9	12.6	0.45
G.B.—M	9– 2	128.8	26.2	12.9	13.1	0.50
R.J.—M	9– 8	134.9	33.4	12.7	14.6	0.44
A.Z.—M	9– 4	137.2	32.2	12.5	11.3	0.35
L.P.—M	9–11	139.0	35.8	12.4	14.8	0.41
C.R.—M	9– 3	134.9	32.4	12.2	15.1	0.46
E.D.—M	9– 7	130.8	28.2	12.0 [1]	11.9	0.42
A.P.—F	9– 7	135.9	31.5	11.7	11.1	0.35
R.H.—M	10– 1	126.8	25.5	11.6	11.2	0.44
Ave. M and F	9– 9	136.1	32.3	12.9	12.6	0.40
Ave. M	9– 4	133.3	30.4	12.7	13.3	0.44
Ave. F	10– 2	139.8	34.7	13.0	11.7	0.34
H.S.[2]	10– 5	135.6	39.0	13.1	11.4	0.29

[1] Later rose to 13–14 area.
[2] For 12-week period.

that will furnish an accurate sample of a subject's customary intake. The food each child ate was weighed on Hanson spring scales to an accuracy of 1 gm. The subjects drank from thermos bottles filled with measured amounts of tap

TABLE 2

The effect of iron administration for 7 months on the red cell counts and hemoglobin values.

		HEMOGLOBIN Grams per 100 ml.			RED CELLS Millions per c.m.m.		
		Spring	Fall	Change	Spring	Fall	Change
0 mg. added iron							
Boys	T.R.	13.8	14.3	+0.5	4.73	4.34	—0.39
	B.C.	13.4	14.1	+0.7	3.97	4.27	+0.30
	J.J.	13.0	13.8	+0.8	4.41	3.97	—0.44
	C.R.	12.2	12.0	—0.2	4.11	3.85	—0.26
Girls	B.Co.	13.2	13.0	—0.2	3.84	3.86	+0.02
	H.S.	13.1	14.2	+1.1	4.51	4.30	—0.21
	A.H.	13.0	13.0	0.0	4.10	3.75	—0.35
Average		13.1	13.5	+0.4	4.24	4.05	—0.19
2 mg. added iron							
Boys	B.H.	13.5	12.8	—0.7	4.45	4.51	+0.06
	G.H.	12.9	12.5	—0.4	3.98	3.86	—0.12
	E.D.	12.0	12.7	+0.7	3.97	4.23	+0.26
	L.P.	12.4	sick		4.40	sick	
Girls	G.Ho.	13.3	13.7	+0.4	4.18	4.18	0.00
	R.C.	13.1	13.4	+0.3	4.46	4.10	—0.36
	B.W.	13.0	13.2	+0.2	3.91	3.79	—0.12
Average		12.9	13.0	+0.1	4.19	4.14	—0.05
4 mg. added iron							
Boys	G.B.	12.9	12.3	—0.6	4.44	4.06	—0.38
	R.J.	12.7	13.3	+0.6	3.81	4.22	+0.41
	A.Z.	12.5	12.9	+0.4	4.15	4.14	—0.01
	R.H.	11.6	11.7	+0.1	3.96	3.78	—0.16
Girls	C.W.	13.6	13.2	—0.4	4.04	3.96	—0.08
	D.S.	13.3	13.1	—0.2	4.00	3.95	—0.05
	A.P.	11.7	12.3	+0.6	4.01	3.86	—0.15
Average		12.6	12.7	+0.1	4.06	4.00	—0.06
Average of all subjects		12.9	13.1	+0.2	4.16	4.05	—0.10

water. Weekly composites were made of 1/10 aliquots of the food weighed on trip scales to an accuracy of 1/10 gm., and 1/10 aliquots of water were added.

The composites were digested with concentrated hydrochloric acid on a steam bath. Samples taken from the digests were wet-ashed. Iron was determined on an aliquot of the ash solution by a modification of Stugart's thiocyanate method ('31), with the use of an Evelyn Photoelectric Colorimeter. All containers were rinsed with dilute hydrochloric acid and triple-distilled water, and all possible precautions taken to avoid contamination.

The results of the iron analyses are shown in table 1. It will be noted that the total intakes ranged from 10.8 to 15.1 mg. with a mean of 12.6 mg. The mode, however, lay in the group below the mean, nine out of twenty-one children having intakes between 11.0 and 11.9 mg. and one being only slightly lower (10.8). In general the intakes of the boys were higher than those of the girls; the respective mean intakes were 13.3 and 11.7 mg. or 0.44 and 0.34 mg. per kilogram. The higher intake of the boys is interesting in view of the fact that they had neither a higher hemoglobin nor a higher cell count than the girls.

That the diet during the 2 weeks of the study was typical not only of the present diet but also of the one that had produced the hemoglobin level of the subjects, was borne out by three lines of evidence.

1. A chemical analysis of the diet of one of the girls for 10 additional weeks from April 6th to June 15th showed daily mean intakes of 10.5, 11.4, 10.9, 11.5, 11.2, 10.8, 11.2, 11.4, 12.5, 11.6 mg. with a mean for all 12 weeks of 11.4 mg. This was very near 11.7 mg., the mean for all the girls of the study.

2. Calculations were made from the dietitian's records of food supplied to each of the three cottages in which the subjects lived, for each month of the year, 1939–1940, including the months in which the 2-week samples were taken. The values for the 2 months in which the study was made were

14.5 and 15.5 mg. per day, values which were similar to those for the other months, and to the yearly average of 15 mg.

3. It happened, also, that records were available from a dietary study made in the same institution in 1932. They were compared with the calculated values found in the present study (1940) to determine whether the iron content of the diet had been the same for the last few years. Both of these computations were made from weights taken at the time the food was served on the plates. The mean for the 1932 group was 12.1 mg. and for the 1940 group, 12.6 mg.

Thus all three lines of evidence showed that the 2-week sample was surprisingly typical of the diet on which the children had been living.

The iron supplementation. The object in giving the iron supplements was to see whether the hemoglobin levels of any of the children would rise. A rise would indicate that the amount of iron originally in the diets was too small. The size of the supplement needed to bring about a rise would furnish information for estimating the minimum requirement.

The children having about the same hemoglobin values and red cell counts and approximately the same weight were arranged in groups of threes. One from each group was given 4 mg. of iron in addition to that in the diet, one 2 mg., and the third child none. These amounts were such that the total iron intakes per day would not be greater than could be obtained from foods. The supplement was given as the iron salt, ferric pyrophosphate. It was mixed with the milk in order to parallel the physical relationship of the iron in foods as nearly as possible. This salt was selected because it had been found by other workers to give good absorption when mixed with milk. This iron therapy was continued for 7 months because the amount of additional iron was so small that a long period was necessary to allow for a slow accumulation of new hemoglobin. At the end of this period, hemoglobin and red cells were again determined by the same methods and under the same conditions as at the beginning.

The results, as given in table 2, show that no significant change occurred in the blood picture, not even in the case of the subjects whose hemoglobin values were a little low. The hemoglobin would have had to rise as much as 1 gm. to be certain of a real change. In only one case was there as great a change as 1 gm. and that child was one of the controls who had no additional iron. The average for the whole group gave no indication of a tendency of the hemoglobin to rise. Only small and insignificant changes were found in the red cell counts. The supplementation, therefore, yielded no information as to the lowest iron intake needed for the formation of a good hemoglobin level but did show that the children were getting at least enough iron before the supplement was added.

The lowest intakes found to be sufficient. Since the real object of the whole study was to find the lowest level of iron intake that would support a good hemoglobin level, the data should be examined with this in mind. The subjects have been arranged in the order of the descending amounts of hemoglobin in table 1. The lowest amount of iron in the diet of a subject with a hemoglobin level of 12 gm. or higher was 10.8 mg. per day. This was but one case. That of six subjects averaged 11.4 mg. per day, which was exactly the average daily intake of the one subject who was studied for 10 additional weeks. It seemed justifiable, therefore, to consider this amount sufficient to cover the needs of children of this age.

Since, however, the children varied in age and size, the per kilogram values may have more significance. If all the subjects with a hemoglobin level of 12 gm. or more are considered, it will be observed that three of the children had good hemoglobin levels on approximately 0.30 mg. per kilogram. Since one of these was the child kept on the experiment for 12 weeks, and the only one showing a significant increase in hemoglobin level, it may be that 0.30 mg. per kilogram is sufficient. However, since these three children were all above the standard weight for children of their age, and would thus have a relatively high total intake on a low per kilogram intake, it might

not be wise to apply this figure to all children. The ten subjects with the lowest intakes were receiving 0.30 to 0.40 mg. per kilogram. Only two of these were boys. However, if two boys could build a good hemoglobin level on this amount, the rest could probably have done so. The mid-value of 0.35 mg. per kilogram would therefore appear to be an adequate allowance. If this value is applied to all the children of the study, it gives an average total iron of 11.3 mg. This is almost identical with the average total (11.4 mg.) of the six subjects with the lowest intakes and good hemoglobin values.

DISCUSSION

Intakes of 11.4 mg. or 0.35 mg. per kilogram were apparently sufficient for the formation of a good hemoglobin level, but were they actually larger than necessary? This question cannot be answered with certainty until accurate studies have been made at lower intakes than these. Nevertheless it is possible to find some information on this point. The view that a lower intake would have sufficed is supported by the study of Davidson et al. ('33) who made a study among large groups of people from the poorer classes in Scotland. Calculations of the iron content of the diets were made from the foods entering the homes. Intakes of iron as low as 5 to 6 mg. "per man" (no doubt less per child) were found among families in which there was no anemia. In a study of 3000 individuals, Davidson and Fullerton ('38), also, found considerable anemia among very young children and women but little among men and children of the early school age. Since these people came from the poorer classes, the diets of the children undoubtedly contained less iron than that found in the diets of the subjects of the present study. The findings of Ross and Summerfeldt ('36) are to the contrary. They made a study on children 5 to 14 years of age living in an orphanage. The average hemoglobin for the group was low (10.2 gm.) although the diet, as calculated, contained 11 mg. of iron. Since the diets of their subjects were not as good as those of the present study and since supplementation with vitamins

B_1 and B_2 improved the hemoglobin of their subjects, it seemed likely that their diets may have been deficient in factors other than iron necessary for hemoglobin formation. Such experimental evidence as exists, therefore, points to the probability that the iron requirement as found by this study is higher than it needs to be.

It may be justifiable to try to get some idea of the minimum requirement by calculations such as those which have been published by Heath and Patek ('37). They estimated the amount of iron which would have to be retained each year in order to furnish enough for a normal increase in tissue and blood from birth to the end of the period of growth (table 3).

TABLE 3

Estimates of the iron requirements of boys[1] from 2 to 11 years based on the retention figures of Heath and Patek.[2]

AGE	WEIGHT	RETENTION PER YEAR	INTAKE PER YEAR	INTAKE SMOOTHED	INTAKE PER DAY	INTAKE PER KILOGRAM
yr.	*kg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
2	13.4	80	800	850	2.3	0.17
3	15.3	92	920	920	2.5	0.16
4	17.3	98	980	980	2.7	0.15
5	19.6	79	790	1050	2.8	0.14
6	22.2	80	800	1120	3.1	0.14
7	24.6	70	700	1180	3.2	0.13
8	27.9	72	720	1250	3.4	0.12
9	30.9	152	1520	1320	3.6	0.12
10	34.5	130	1300	1390	3.8	0.11
11	37.6	137	1370	1450	4.0	0.11

[1] Retentions for girls are approximately the same.

[2] Heath, C. W., and A. J. Patek, Jr. 1937. The anemia of iron deficiency. Medicine, vol. 16, p. 279.

In order to find the size of the intake necessary to obtain a given retention, reference has been made to the percentage of retention found in balance experiments on children by the method of iron analysis used in this study.

Ascham ('35) and Oldham et al. ('37) found retentions of about 10%. Porter ('41) found retentions of about 20%. With the use of the polarographic method of analysis, Macy ('41) reported retentions of approximately 10%. Since it

is safest to use the percentage which would require the highest intake, a retention of 10% has been assumed for purposes of calculation. The required retentions as computed by Heath and Patek have then been multiplied by 10 to find the intake necessary to produce them and the values have been smoothed by inspection. By this method of calculation, a child of 10 would need a total of 3.8 mg. per day or 0.11 mg. per kilogram (see table 3). The intakes of all the subjects of the present study were much higher than this.

However, children of these ages need not only enough iron for maintenance and growth but also enough to allow the storage of iron in the liver and other storage depots. If the values given in table 3 are increased by 100% on the assumption that most of the stores must be accumulated in these years, the intake would be 7.6 mg. or 0.22 mg. per kilogram at 10 years. Thus even with allowances of 90% for loss in absorption and 100% for storage, the calculated iron requirement is still below the intakes found in the present study. It is true that figures such as these calculated ones, backed by no experimental work, cannot be used with much confidence but they do perhaps give some indication that the children of this experiment were getting even more iron than they needed for maintenance, growth and storage.

It may be of interest to compare the intakes of 0.35 mg. per kilogram found sufficient for the children of this study with those previously found for preschool children by means of the balance experiment. The requirements suggested have ranged from 0.60 mg. per kilogram (Rose et al., '30; Daniels and Wright, '34; Ascham, '35) to 0.48 mg. (Leichsenring and Flor, '32). However, since Porter ('41) has recently reported good retentions on intakes averaging 0.31 mg. per kilogram, it may be that the upper value of 0.60 mg., which has generally been accepted, is higher than it needs to be.

SUMMARY

The purpose of this study was to find the iron requirement of children of the early school age. This was to be accom-

plished by finding the lowest iron intake which would support a good hemoglobin level.

Hemoglobin determinations were made on twenty-one children 8 to 11 years of age. Of these, nineteen had hemoglobin levels of 12–14 gm., the range considered normal.

The iron intakes were found by making a chemical analysis of the diet of each child over a 2-week period. An examination of the intakes indicated that good hemoglobin levels could be maintained on 11.4 mg. per day or 0.35 mg. per kilogram when the rest of the diet was adequate.

That a higher intake was not needed was shown by the fact that the hemoglobins of children given 2 or 4 mg. of iron daily over a 7-month period did not rise. It is possible, also, that lower intakes, had they been tried, might have been found to serve just as well.

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THE ASCORBIC ACID REQUIREMENTS OF CHILDREN

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ONE FIGURE

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The practical problem of estimating the adequacy of the dietary with respect to ascorbic acid depends upon the knowledge of two factors: the ascorbic acid requirements and the amounts of ascorbic acid that various foods contribute after being prepared for consumption.

Since the development of chemical methods for ascorbic acid analysis it has been shown that in most normal subjects both the ascorbic acid value of the blood plasma and the amount excreted in the urine after a test dose reflect the previous dietary intake (Hawley, Stephens and Anderson, '36; Sendroy and Schultz, '36; Abbasy, Harris, Ray and Marrack, '35; Abt and Farmer, '36; Wright, Lilienfeld and MacLenathen, '37). Numerous reports have appeared in which either urine or blood analyses have been used to determine the state of ascorbic acid nutrition (Minot, Dodd, Keller and Frank, '40; Parran, '40). In a few cases studied, mostly those on adults, blood plasma determination or urinalysis has been supplemented by a determination of the intake in order to gain information on requirements (Belser, Hauck and Storvick, '39; Todhunter and Robbins, '40; Goldsmith, Ogaard and Gowe, '41; Smith, '36; Ralli, Friedman and Sherry, '39). However, the important problem of the ascorbic acid requirements for children has received less attention. Everson and Daniels ('36) in reporting a study on three pre-school boys,

considered 6.0 to 7.5 mg. per kilogram of body weight to be required daily for optimal retention. Widenbauer ('37) found that a daily intake of 22 mg. was a satisfactory level for a child $2\frac{1}{2}$ years old. Hathaway and Meyer ('41) reported 31 mg. daily as sufficient to maintain "tissue saturation" in four cases from 4 to 5 years of age. These studies were based on the procedure of finding the individual daily ascorbic acid intake which would cause most of a subsequent test dose to be excreted in the urine. The individual intake was established either by direct food analysis or by the use of pure ascorbic acid.

Determinations of requirements by this method must, of necessity, be done on a small group of patients who are kept under the strict conditions of metabolism studies. Such careful physiological studies are essential, and undoubtedly when sufficient data of this type have been accumulated it will be possible to determine the requirements with considerable precision. However, in the application of such knowledge to the practical problems of human feeding, there will still exist the uncertainties involved in meeting these requirements with food. These uncertainties are many in the case of ascorbic acid, owing to the variable losses of this unstable substance, depending upon the type of food, conditions of growth, and the methods of marketing and preparation (Bessey, '38 b). The important practical problem in ascorbic acid nutrition is: How much of what kinds of food as usually prepared and consumed is necessary in order to be sure that the ascorbic acid requirements (whatever they may be) are adequately met? Because of the difficulties mentioned above, it seemed important to establish the ascorbic acid requirements for children under home conditions in terms of the food usually consumed, and by methods applicable in a nutrition clinic.

In this investigation, the blood plasma ascorbic acid levels of ninety-three healthy city children who were receiving variable amounts of ascorbic acid in their food, have been determined. Requirements were estimated by plotting these analyses against the dietary intakes as derived from dietary

histories considered unusually reliable, and locating the minimum intake which would give the average maximum post-absorptive blood plasma level.

EXPERIMENTAL

Children attending the medical-nutrition clinic of the Forsyth Dental Infirmary were used for the study. This clinic provides periodic medical examinations and nutrition conferences for the children of the Infirmary as a means of improving dental conditions and promoting bodily health. Children are frequently under supervision for a period of several years. Since only those children who are financially eligible for clinic care are admitted to the Infirmary, the families represented in the study were in the lower income brackets of the community. Many were receiving assistance from public or private relief agencies.

By the system of including in the study the first few children who appeared on each clinic day, a representative cross-section was selected for blood determinations. The age range was from 5 to 13 years inclusive and approximately an equal number of boys and girls were used. Since all the children selected were given medical examinations on the day of the test, it was possible to exclude those who showed evidence of colds or other acute infection.

Specimens of blood were obtained by ear puncture with a Bard-Parker blade 4 to 24 hours after the last intake of a potent source of ascorbic acid (citrus fruits and tomatoes) and the analysis was made immediately. In many cases, subsequent blood plasma determinations were made after a lapse of a month or longer.[1]

The microphotoelectric indophenol method described by Mindlin and Butler ('38) with the modifications in manipula-

[1] Blood plasma analysis is preferred to urine determinations, especially for an out-patient clinic, since a satisfactory specimen may be obtained in a few minutes. One objection to its use for large groups of children has been the necessity of a venous puncture in order to obtain sufficient blood for an accurate analysis. This difficulty may be avoided by the use of the microphotoelectric technique, which requires only 0.25 cc. of blood. This amount may be easily obtained from the ear lobe without frightening the child.

tion and calculations suggested by Bessey ('38 a) for the macro method, was used for analysis. The acid filtrates and dye were measured in Lang-Levy pipettes (Levy, '36).

The values for the consumption of ascorbic acid were based on dietary histories secured by personal clinic interviews with the mother and child. The nutrition teaching that is regularly carried on in the clinic conferences was continued as usual, but for the purpose of the survey special attention was directed to the ascorbic acid consumption of the children from whom a blood sample had been taken. The mother and child were questioned minutely as to the food eaten during the 24-hour period previous to the clinic visit. Habits with respect to the usual consumption of the following foods were recorded: citrus fruits (oranges, grapefruit, tangerines and lemons) fresh or canned; citrus fruit juices, fresh or canned; tomatoes, fresh or canned; other fresh fruits and vegetables; and other canned or cooked fruits and vegetables. Detailed consideration was also given to the many factors which influence the child's consumption of these foods, such as family habits of purchasing, preparing and serving, food expenditures, meal planning, individual food preferences, and child management.

An estimate was made of the amount of ascorbic acid consumed daily by each child. The confidence with which the dietary data are regarded rests partially upon the fact that they were collected by a nutrition specialist in a clinic in which nutrition procedures were well established. Another significant feature is that many of the children had been under supervision for several years. In these cases, the records indicated clearly the response of the mother and child to clinic teaching and the validity of their statements. The trend of the child's dietary habits could therefore be appraised with considerable assurance.

Any record that contained an inconsistency or a suspicion of unreliability was discarded for the purpose of this report. From 180 cases, 93 with dietary records that were regarded as unimpeachable were selected.

Upon studying the dietary records, it was found that either citrus fruits or tomatoes constituted the principle year-around source of ascorbic acid for these children. The amount of ascorbic acid obtained from other sources appeared to be so small as not to influence the blood value, at least in the zone of concentrations found after liberal intakes — a point which will be discussed later. The estimate of the intake was, therefore, based on the consumption of citrus fruits and tomatoes only. Oranges, grapefruit and lemons were assumed to be equivalent. Tomatoes and tangerines were considered to contain about one-half as much vitamin C as oranges. The dietary estimates were made in terms of ounces of orange juice and the ascorbic acid calculated at 15 mg. per ounce.

CONCLUSIONS AND DISCUSSION

Figure 1 shows the results of the blood plasma analysis for each level of ascorbic acid intake expressed as milligrams per day. One hundred and thirty-nine analyses on ninety-three cases are represented, since in several individual cases more than one determination was made.

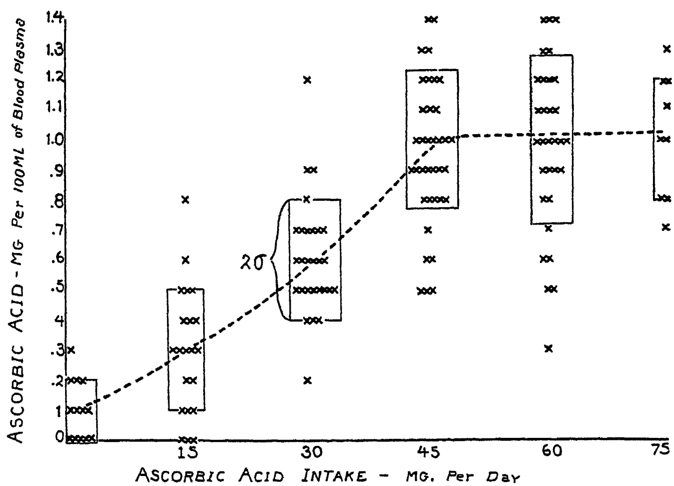

Figure 1

It will be noted that there is considerable scattering of points at any one level. This indicates a rather wide individual dispersion in the effect of a given quantity of orange juice on the blood plasma ascorbic acid level, and therefore suggests the caution which is necessary in evaluating the state of ascorbic acid nutrition of an individual by this method; but, in spite of this variation, there is a clear tendency for the points to concentrate around certain values.

It is an interesting fact that a curve drawn through the average values duplicates the curves that have been obtained by one of us (O.A.B.) when repeated analyses have been made on normal individuals on gradually increasing doses of pure ascorbic acid. This agreement in the data collected by two different methods gives support to the validity of the data presented. In figure 1, it will be observed that up to the level of about 3 ounces of orange juice, the ascorbic acid value of the blood plasma shows a gradual increase as the intake of ascorbic acid increases. Beyond this point the increase in value is slight, if any. Further support is given to the reliability of the data by the fact that this maximum concentration is the value found by kidney threshold investigations (Ralli, Friedman and Sherry, '39).

The data presented indicate that 3 ounces of orange juice per day, or its equivalent, plus the small amount of ascorbic acid obtained in other foods, which probably totals not more than 10 mg. per day in this group of children, is sufficient to produce an optimal post-absorptive plasma ascorbic acid concentration for the average child aged 5 to 13 years. Assuming orange juice to contain 15 mg. of ascorbic acid per ounce, the requirements may be placed at 40 to 50 mg. daily. It is true that on this level of intake some cases do not reach the average optimal blood plasma value. However, the number of such cases falling below the standard seems also to be about the same on both the 4-ounce and the 5-ounce intake. That this low level may be characteristic of the individual is suggested by the fact that reanalyses several weeks apart checked each other almost without exception within the limits of the method.

This finding also corresponds with the experience of one of us (O.A.B.) with certain other individuals who fail to reach the expected fasting blood plasma level even when the intake of pure ascorbic acid is very high.

When not only the citrus fruits and tomatoes but also all other fruits and vegetables were included as sources of ascorbic acid, no correlation was apparent between the dietary evaluation and the blood plasma values. Since there is no evidence to indicate that ascorbic acid from sources other than citrus fruits and tomatoes is poorly utilized, we must interpret this lack of correlation as meaning that our estimates of the amounts of ascorbic acid contributed by fruits and vegetables other than the citrus fruits and tomatoes were incorrect. We believe that this difficulty often arises from the use of food-value tables for the ascorbic acid content of foods which are invariably subject to large and unpredictable losses during marketing and preparation.

That the amount of ascorbic acid obtained from sources of low potency has little influence on blood plasma values is indicated also by the fact that when children on the same levels of citrus fruit intake were divided into two groups, namely those receiving the largest amount of other types of fruit and vegetables and those receiving the smallest amounts, there was no difference in the average blood plasma values.

Eight children of the group studied consumed practically no citrus fruits or tomatoes for several months during the study. The blood plasma values of these children were either zero or near zero on every analysis. Four cases failed to show ascorbic acid in the blood plasma at any time, yet clinical evidences of scurvy were absent. This fact seems to indicate that for such a group the average daily consumption of ascorbic acid from sources other than citrus fruits and tomatoes is sufficient to prevent scurvy, but insufficient to give satisfactory blood plasma levels. The absence of scurvy in these cases confirms several recent reports (Butler and Cushman, '40; Crandon, Lund and Dill, '40; Milam and Wilkins, '41) that zero or low plasma levels are not necessarily indicative of this

disease. Under such conditions, the tissues although probably handicapped, may receive enough ascorbic acid to prevent the development of pathology.

We do not wish to leave the impression that food sources other than citrus fruits and tomatoes are unimportant or unable to supply the ascorbic acid required to produce satisfactory plasma values. Obviously a sufficient intake of any ascorbic-acid-containing food should be adequate; and undoubtedly during certain seasons or in certain regions the intake of such products as strawberries and raw green leafy foods may be great enough to be sufficient. However, the average city child does not regularly consume enough of these foods to reach a desirable intake of ascorbic acid.

Our findings indicate that an estimation of the intake of citrus fruit and tomato serves as a very simple and satisfactory practical means of evaluating a diet for vitamin C when one is concerned with optimal nutrition, and just as the regular consumption of milk is considered a practical necessity to assure adequate calcium, so the regular use of citrus fruits or tomatoes is the most practical and reliable means of assuring the optimal ascorbic acid intake.

The point is often raised as to whether the placement of the requirement at the amount which produces the plasma concentration usually found after liberal intake may not be too high a standard. It seems likely that this level is above the optimal physiological requirement and that it involves some waste; but until methods are available for fixing more exactly the value of the physiological optimum, it would seem to be a good nutritional practice to recommend a daily intake of ascorbic acid that is within the range of the physiological maximum.

SUMMARY

Blood plasma ascorbic acid analysis on ninety-three healthy city children whose ascorbic acid intake is evaluated from thorough dietary histories is presented.

Eighty per cent of the children who received 3 ounces or more of orange juice daily or an equivalent amount of ascorbic

acid in citrus fruits and tomatoes maintained a blood plasma level of ascorbic acid indicating a liberal intake. Those who received less than 3 ounces showed blood plasma values which were correspondingly lower. There were a few individuals whose blood plasma values remained definitely low in spite of a regular intake considered adequate for the average child.

The amount of ascorbic acid consumed daily from sources other than citrus fruits and tomatoes, that is, other fruits and vegetables, was insufficient in this group to influence the blood plasma values. This finding emphasizes the necessity of a regular consumption of citrus fruits or tomatoes if an optimal post-absorptive plasma value is to be maintained. Furthermore, it suggests that in taking nutritional histories the evaluation of the citrus fruit and tomato intake is a simple, practical, and accurate means of determining ascorbic acid consumption, at least as far as the optimum range is concerned.

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A CONVULSIVE SYNDROME IN YOUNG RATS ASSOCIATED WITH PYRIDOXINE DEFICIENCY

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The use of the U.S.P. method of assay for thiamine [1] as a satisfactory and accurate procedure is now well established. In studies designed to demonstrate that the U.S.P. diet is complete in all respects except for thiamine (Kline, Tolle and Nelson, '38) it was found that animals reared to maturity on the thiamine test diet supplemented with crystalline thiamine were capable of normal reproduction. These and subsequent studies, however, revealed the inability of such animals to maintain their young normally through the period of lactation. A syndrome occurring in the young during the suckling stage was characterized by frantic running about the cage, accompanied by loud cries and followed by convulsive seizures. This condition was not cured by administration of crystalline thiamine, yeast, or liver extract. It was demonstrated, however, that the convulsive syndrome in the suckling young bore no relationship to the nutritional state of animals being used for thiamine assay and had no influence upon results of the assay method conducted in accordance with the specified U.S.P. procedure. With animals employed for this assay the administration of relatively large doses of riboflavin, pyridoxine, nicotinic acid, pantothenic acid or choline, in addition to the usual curative dose of thiamine standard, was found to have

[1] The Second Supplement to the Pharmacopoeia of the United States of America, Eleventh Decennial Revision, p. 129.

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no effect upon the length of curative response to vitamin B_1 (Kline, Hall and Morgan, '41). Only during the period of lactation was there any evidence of deficiency. Further study both of the deficiency syndrome in the young and its cure is the subject of this presentation.

EXPERIMENTAL

These studies involved the observation of a number of rats throughout the period of reproduction and lactation, and of litters of young produced under several experimental conditions. The females used were from two sources: stock animals changed to the experimental diets during the period of pregnancy, and animals reared on the experimental diets from weaning through maturity and reproduction. The animals were kept on raised screens in the usual manner except during a short period preceding parturition and the 15 days following. During this time they were caged on screens resting directly on sawdust. Diet and water were available ad libitum. Records were kept of birth of litters, of weights of mothers and young taken at regular intervals, and of observations of condition of the animals throughout the suckling stage. The composition of the diets fed is given in table 1 and in table 2 is indicated the number of animals studied on each of these diets.

Twenty adult females were placed on diet 22E, a ration which had been demonstrated to be adequate for normal growth and reproduction. This is a ration used in the laboratory for thiamine assay which has been supplemented with 200 μg. each of crystalline thiamine and riboflavin per 100 gm. of diet. Thirteen of the twenty females studied had been reared from weaning to maturity on this diet, two were stock animals changed from the stock diet during pregnancy and five were second-generation animals reared on diet 22E.

In an attempt to influence the symptoms which developed in the young born to these females a number of supplements were administered orally to both the young and mother as indicated in table 2. In several litters stock colony young of the same

age were substituted for a part of the litter of mothers on diet 22E and the experimental young in turn were given to the stock females retained on the stock diet. Treatment of the mother included administration of alpha-tocopherol, administration of pyridoxine, both by oral feeding and supplementation of the diet, and modification of the diet to omit the autoclaved peanuts. This modified diet is referred to as diet 23E.

TABLE 1

Composition of diets.

INGREDIENTS IN DIET	DIET 22E	DIET 23E	DIET 64B	DIET 65	DIET 69
	Grams per 100 gm.				
Casein [1]	18	18	18	18	18
Salts U.S.P. no. 1	4	4	4	4	4
Cod liver oil	2	2	1	1	
Sucrose	60	70	72	72	78
Autoclaved yeast	5	5			
Autoclaved peanuts	10				
Liver extract (irrad.)			1		
Liver extract (sulphite-treated)	1	1			
Corn oil			5	5	
	Micrograms per 100 gm.				
Thiamine	200	200	200	200	200
Riboflavin	200	200	300	300	300
Calcium pantothenate			500	500	500
	International Units per 100 gm.				
Vitamin D (irrad. ergosterol)					300
Vitamin A concentrate					300

[1] The casein was either acid reprecipitated or sulphite treated.

Eight adult females were placed during pregnancy on diet 64B, a more highly synthetic mixture containing purified sources of protein and carbohydrate and including 1% liver extract of the type ordinarily used as a source of the pernicious anemia factor. This ration was deficient in pyridoxine. The animals placed on this diet were a stock female trans-

ferred from the stock ration during pregnancy and seven animals which had been reared to maturity on diet 64B supplemented with pyridoxine.

TABLE 2

Summary of animals, diets and supplements.

TOTAL NUMBER OF ADULT FEMALES	DIET	TOTAL NUMBER OF LITTERS	TOTAL NUMBER OF YOUNG	MODIFICATIONS OF DIET		RESULTS
				Female	Young	
20	22E	37	360	None	None	Failure of young
				Alpha-tocopherol	None	Failure of young
				Peanuts omitted	None	Failure of young
				Pyridoxine	None	Normal young
				None	Alpha-tocopherol	Failure of young
				None	Yeast	Failure of young
				None	Ca pantothenate	Failure of young
				None	Corn oil	Failure of young
				None	Suckled by stock ♀	Normal young
				None	Pyridoxine	Normal young
8	64B	15	131	None	None	Failure of young
				None	Corn oil	Failure of young
				Pyridoxine	None	Normal young
2	65	2	20	None	None	Failure of young
2	69	3	39	None	None	Failure of young
				Pyridoxine	None	Normal young

Two adult stock females were transferred during pregnancy to each of diets 65 and 69, both of which were similar to diet 64B except for the omission of liver extract. Each ration was deficient in pyridoxine.[2] In diet 69 a concentrate of vitamins A and D was used in place of the cod liver oil and the corn oil was omitted.

RESULTS

A general summary of the results of these experiments is shown in table 2. Table 3 gives more detailed information on typical animals showing results which are representative of those obtained throughout the study.

[2] The terms vitamin B_6 and pyridoxine are synonymous and are used interchangeably in this presentation.

On each of the diets 22E, 64B, 65 and 69 normal reproduction was obtained but failure of the young was noted during the lactation period. Characteristic symptoms developed in the young usually between the eleventh and eighteenth days. These animals suddenly appeared emaciated, showed tremor, uttered sharp cries apparently due to pain, exhibited a series of running fits accompanied or followed by convulsions and in most cases by death on the same or following day. Even the young from stock females placed on these experimental diets a day or two before parturition exhibited such symptoms. The development of the deficiency was therefore not dependent upon maintenance of the female on any one of these diets for an extended time before birth of the young.

Normal stock colony young suckled by mothers on diet 22E from the first day after birth developed these same deficiency symptoms. In these cases, however, the onset of the symptoms was delayed and a small proportion of the animals survived. On the other hand, it was found that young from diet 22E females when placed with normal, lactating stock females receiving the stock ration developed normally in every respect.

That lack of vitamin E was not the cause of the symptoms was demonstrated by feeding synthetic *dl*-alpha-tocopherol at different times to both the female and the young, after which the characteristic condition still occurred. Neither was there any alleviation of the symptoms by omitting the autoclaved peanuts from diet 22E. This was done to eliminate any possible destruction of vitamin E that might have occurred as a result of rancidity in this material. Calcium pantothenate in large doses administered orally to the young did not prevent the occurrence of the symptoms. The effect of yeast fed after the onset of the condition was questionable. Because of the rapid development of the symptoms it is possible that the yeast was not administered sufficiently early to allow effective absorption from the alimentary tract. Corn oil, given to the young, did not appear to have any marked effect except to prolong slightly the survival period. The symptoms were rapidly cured or prevented when pyridoxine was administered orally

TABLE 3

Records of typical animals.

Description of female		Diet of female	Litter number	Number of young born[1]	Portion of the litter receiving supplement: Supplement
Reared to maturity on diet 22E	1	22E	1	8	
		22E	2	8	Suckled by stock ♀ on stock diet
		Stock	3	9	
		22E + B_6 daily	4	4	
	2	22E	1	11	Alpha-tocopherol on 13th day
		23E + alpha tocopherol	2	5	
		22E	3	9	Suckled by stock ♀ on stock diet Yeast 16th day (after tremor) B_6 (after convulsions)
		22E + 40 μg. B_6 daily	4	11	
	3	23E + alpha tocopherol	2	7	
		22E	3	6	Suckled by stock ♀ on stock diet B_6 15th day (after convulsions) Yeast 16th day (after convulsions)
	4	22E	1	9	Alpha-tocopherol on 9th day
		22E	2	11	Ca pantothenate 13th and 15th d
	5	22E	2	8	Corn oil 13th day (after convulsions)
	6	22E	1	9	B_6 (10 μg. daily 3rd–6th day then 20 μg. daily to weaning)
	7[2]	22E	1	5	
Pregnant stock ♀	8	22E	1	10	B_6 (80 on 18th and 22nd day—after convulsions)
Reared to maturity on diet 64B + vitamin B_6	9	64B + 200 μg. B_6 per 100 gm. diet	1	8	
		64B + 200 μg. B_6 per 100 gm. diet	2	6	
		64B from 2 days before parturition	3	12	
		64B + 200 μg. B_6 per 100 gm. diet	4	10	
	10	64B + 200 μg. B_6 per 100 gm. diet	1	12	
		64B from 2 weeks before mating	2	10	
		64B + 200 μg. B_6 per 100 gm. diet	3	11	
Pregnant stock ♀	11	64B from 4 days before parturition	1	8	0.1 cc. corn oil 15th, 17th and 22nd day
		64B + B_6 from 2 weeks before mating	2	5	
Pregnant stock ♀	12	65 from 2 days before parturition	1	8	
Pregnant stock ♀	13	69 + 40 μg. B_6 daily from 6 days before parturition	1	15	
		69 from 7 days before parturition	2	10	

[1] Number of young studied is less than number born in many litters because of usual death occurring soon after birth or reduction of size of large litters depending on condition of mother

TABLE 3

Records of typical animals.

PORTION OF THE LITTER RECEIVING SUPPLEMENT		PORTION OF LITTER RECEIVING NO SUPPLEMENT		STOCK YOUNG SUCKLED BY FEMALE ON EXPERIMENTAL DIET	
of young	Results	Number of young	Results	Number of young	Results
		8	Died 11th–13th day		
2	Normal	2	Convulsions, died 17th–18th day	2	Convulsions — survived
		8	Normal		
		4	Normal		
2	Died 14th and 15th day	8	Died 12th–13th day		
		5	Died 15th–17th day		
4	Normal	2	Convulsions, 1 died 15th day	4	Convulsions — survived
1	Survived				
1	Survived				
		10	Normal		
		4	Died 13th day		
3	Normal			5	Convulsions — died
1	Survived — normal				
2	Died				
4	Died 11th–13th day	4	Died 11th–13th day		*
4	Died 17th day	4	Died 13th–17th day		
4	Died 15th–17th day	2	Died 13th day		
8	Normal				
		5	Died 12th–19th day		
2	Survived — normal	8	6 died 17th–24th day		
		8	Normal		
		6	Normal		
		9	Died 19th–25th day		
		10	Normal		
		8	Normal		
		10	Died 13th–17th day		
		9	Normal		
1	Died 36th day	7	6 died 15th–21st day		
			7th died 41st day		
		5	Normal		
		8	6 died 15th–22nd day		
		10	Normal		
		10	Died 17th–47th day		

* Second generation on diet 22E.

to the young. The diet of the mother supplemented with pyridoxine was also found to be effective in preventing the onset of the symptoms. Such administration of pyridoxine to either mother or young allowed normal development of the young.

Pregnant females placed on diet 64B a short time before parturition were found to produce normal litters. All of the young, however, developed pyridoxine deficiency characterized by the convulsive symptoms. None of these animals survived the deficiency state unless the diet was supplemented with pyridoxine. Hemoglobin determinations on the blood of young animals in the acute stage of the deficiency indicated a normal hemoglobin level. By alternately feeding diet 64B and 64B supplemented with 40 µg. of pyridoxine daily in succeeding pregnancies to the same female it was possible to produce at will the characteristic deficiency in the young in one litter followed by a normal litter in the next reproductive cycle. The same results were obtained by alternating in the same manner the stock colony diet with diet 22E. It was possible as well to produce these convulsive symptoms in the young borne by females transferred during late pregnancy from the stock diet to diets 65 and 69. These were diets in which the liver extract had been omitted.

It was found that the occurrence of the deficiency symptoms was in some cases somewhat delayed in the young from stock females placed on the experimental diets after pregnancy was well advanced. It was also noted that a few of the young from such stock females changed to diet 22E, after exhibiting characteristic deficiency symptoms, survived on the unsupplemented diet. This would serve to indicate that diet 22E contains a borderline level of pyridoxine. Those animals which survived and which were maintained to maturity on diet 22E were found capable of normal reproduction but their young failed during the lactation period. The litters were not as large and the care of the young not as good as in the parent group and the young of five such animals all exhibited the characteristic convulsions and all failed to survive.

To determine whether these symptoms were a specific result of pyridoxine deficiency in the young, pregnant females were placed shortly before parturition on diet 65 modified to contain pyridoxine but deficient in riboflavin or pantothenic acid. Pantothenic acid deficiency, which occurred in the young between the tenth and twentieth day, was characterized by appearance of watery eyes and by sudden respiratory failure with collapse and death. The riboflavin-deficient young, on the other hand, became denuded and, although very much stunted in growth, were able to survive 5 to 6 weeks before extreme emaciation and death occurred. The symptoms exhibited by the young on either of these diets were in no way similar to those resulting from pyridoxine deficiency.

DISCUSSION

The authors are unaware of any previous report of characteristic symptoms of pyridoxine deficiency involving a paralytic or convulsive type of response in young rats of suckling age. This condition is readily reproducible and it has been demonstrated that pyridoxine is specific in its cure and prevention. Chick, Macrae, Martin and Martin ('38) observed epileptic fits in young pigs reared on synthetic diets and found that the condition could be cured with addition of the eluate fraction of yeast known to contain vitamin B_6. In 1940 a report from the same laboratory (Chick, El-Sadr and Worden) described occurrences of epileptiform fits in mature rats maintained for long periods on a diet deprived of vitamin B_6. Jukes ('39) reported the occurrence of spasmodic convulsions in chicks receiving vitamin B_6-deficient diets. Convulsions have been observed in dogs and have been attributed to a lack of vitamin B_6 (Fouts and coworkers, '38). It was interesting, in view of the occurrence of a microcytic hypochromic anemia in dogs on vitamin B_6-deficient rations (Fouts and coworkers, '38, '39), that our preliminary examination indicated no reduction in hemoglobin level in young rats during the acute stage of the deficiency. Jukes ('39) reported normal differential blood-cell counts for vitamin B_6-deficient

chicks. Woolley ('40) described a convulsive type of syndrome in mice fed highly purified diets which was alleviated by administration of pantothenic acid. The studies reported here, demonstrating a lack of relationship of pantothenic acid to the convulsive syndrome in suckling rats, illustrate further metabolic differences in this regard.

A neuromuscular syndrome in young rats, caused by a lack of alpha-tocopherol, has been reported by a number of investigators since the first description by Evans and Burr ('28). Our use of alpha-tocopherol in attempts to find a curative agent for the convulsive syndrome gave negative results and led us to conclude that we were not dealing with a vitamin E deficiency although the possibility was suggested that the alpha-tocopherol administered was not assimilated. To avoid a possible destructive action of rancidity, as shown by Commings and Mattill ('31) and discussed by Mattill ('38), the autoclaved peanuts were omitted from diet 22E without effect. In view of a recent report of MacKenzie et al. ('41), our levels of alpha-tocopherol were sufficient to counteract any dystrophic effect (Davis, Maynard and McCay, '38) of cod liver oil.

A relationship between fat metabolism and pyridoxine requirement has been suggested by a number of investigators. Using small amounts of corn oil, Quackenbush et al. ('39) obtained cure of a rat acrodynia similar to that described by György ('35). We were unable to demonstrate any curative effect of corn oil fed to young in the acute stage of the convulsive syndrome, although the survival period was slightly increased in some animals. These results furnish little evidence of a fat-sparing action on pyridoxine.

There was no observable injury to females maintained on diets devoid of pyridoxine during the period of pregnancy and lactation. After the loss of two or more litters from pyridoxine deficiency it was possible for such mothers to rear normal litters on these diets supplemented with pyridoxine. This dietary factor was readily transmitted to the young during lactation since inclusion of this factor in the

diet of the mother proved to be as effective as direct administration to the suckling young.

The young animals receiving pyridoxine directly developed and grew normally during the suckling stage, demonstrating that the quality, rather than quantity, of milk secreted was affected. This would indicate that pyridoxine can in no sense be designated a lactation factor. That small reserves of this factor are available to the animal on an unrestricted diet is brought out by the difficulty of the lactating female in secreting milk of a normal pyridoxine content after a relatively short period on a diet subminimal in pyridoxine, as well as by the rapidity with which the deficiency syndrome appeared in stock young transferred to mothers on a pyridoxine-deficient diet. The data presented are insufficient to indicate the minimum requirement of pyridoxine for suckling young or for secretion of milk of normal composition.

SUMMARY

A description is given of a convulsive syndrome which occurred in young rats during the suckling stage when being nursed by mothers on a diet considered to be satisfactory from the standpoint of growth and reproduction. This syndrome was cured or prevented by administration of crystalline pyridoxine and, by the use of suitable diets, was demonstrated to be a specific result of pyridoxine deficiency.

Rapid depletion of pyridoxine reserves was obtained in suckling young given to deficient mothers and lactating females transferred from a stock diet to one deficient in pyridoxine.

No injury was observed in females maintained on pyridoxine-restricted diets through the period of pregnancy and lactation. Under such conditions lactation was not impaired although the pyridoxine content of the milk was reduced to such extent that the convulsive syndrome developed in the young.

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ANTIRACHITIC PROPERTIES OF A.T. 10 FOR THE RAT AND CHICKEN

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The common assumption is that dihydrotachysterol is the active component of A.T. 10 (Antitetanisches Präparat Nr. 10). However, since there is no certainty as to the exact composition of this product we shall follow the example of Shohl and Farber ('41) and refer to the material under discussion simply as A.T. 10.

Until recently, most of the experimental work on A.T. 10 was carried out in Germany. Early literature pertaining to this subject has been adequately reviewed by Albright, Bloomberg, Drake and Sulkowitch ('38). The first investigations with A.T. 10 revealed its power to increase serum calcium and relieve tetany after removal of the parathyroid glands. Most of the original investigators did not find A.T. 10 to be antirachitic. Harnapp ('35) was one experimenter who claimed that A.T. 10 had an antirachitic effect.

Albright and collaborators ('38, '39) published a series of clinical investigations comparing the effect of vitamin D_2, parathyroid extract, and A.T. 10 on calcium and phosphorus metabolism in idiopathic hypoparathyroidism, and in rickets. They concluded that A.T. 10 resembled vitamin D in its action by increasing calcium absorption from the gut and phosphorus excretion in the urine. But since it markedly raised serum calcium in the hypoparathyroid patients, and increased urinary phosphates more than did vitamin D, the authors decided that its action more nearly resembled that

of the parathyroid hormone. They suggested that possibly the reason A.T. 10 had not been found to be antirachitic was because of the increase in phosphorus excretion in the urine which it caused.

Recently, Shohl and Farber ('41) reported that they had confirmed the results of Harnapp and found A.T. 10 to be antirachitic for rats. In their studies the prophylactic type of feeding tests was employed and a high calcium, low phosphorus diet was used. They compared the action of vitamin D_2 and A.T. 10 and concluded that, under the conditions of their experiments, A.T. 10 was one four-hundredth as effective as vitamin D_2 in the curing of rickets and was five times as toxic.

With the appearance of the studies by Albright et al. the question arose in our laboratory as to what effect A.T. 10 would have on the serum phosphatase when administered to chickens. Correll and Wise ('38) have demonstrated that certain antirachitics are more efficient than others in maintaining a low serum phosphatase level in chicks. A substance with little or no vitamin D activity would not keep the serum phosphatase content normal but would permit the values to rise. In regard to the relation of parathyroid hormone to serum phosphatase, it has been reported that in the dog (Cantarow, Brundage and Housel, '37) and in man (Gutman, Tyson and Gutman, '36) the concentration of this enzyme is augmented by administration of parathyroid extract or by clinical hyperparathyroidism. Thus, if there was found to be an elevated serum phosphatase after administration of A.T. 10 to chicks it would be a further indication that A.T. 10 more nearly duplicated the action of parathyroid extract than that of an antirachitic agent.

The dual assay for vitamin D, with both rats and chickens, has become a recognized physiologic technique for differentiating between antirachitic substances. As a result of the paper by Shohl and Farber ('41), it was decided to determine what vitamin D potency A.T. 10 might indicate when assayed with rats by the standardized U.S.P. XI ('39) procedure. Using

the rat assay as a measure of antirachitic strength, we then decided to compare the response of the chick to various levels of vitamin D from cod liver oil, vitamin D_2, and A.T. 10 as manifested by body weight, bone ash, serum calcium, phosphorus and phosphatase.

The results obtained from these rat and chick investigations are submitted in this paper.

RAT EXPERIMENTS AND RESULTS

The rat assays to determine the antirachitic activity of A.T. 10 were carried out in a routine procedure conforming exactly to the U.S.P. XI ('39) technique for the assay of vitamin D. Final interpretations were made on the basis of the line test as described by Bills, Honeywell, Wirick and Nussmeier ('31). The antirachitic potency reported for A.T. 10 in this paper was based on these histologic readings, but in addition, in all but the first experiment, ash determinations for each group were made on pooled left tibiae, alcohol- and ether-extracted and moisture free. Also a pooled sample of blood was collected from each group and the serum calcium values obtained using the procedure of Clark and Collip ('25).

Materials investigated were diluted with olive oil so that a definite number of drops supplied the desired daily supplement of active ingredient.

We used commercial preparations as sources of vitamin D_2 [1] and the A.T. 10.[2] The daily doses indicated in the tables represent the weight of active principle fed, calculated from the label statement for each product.

In the first experiment, table 1, we found vitamin D_2 to assay 40,000,000 I.U. per gram (group 89) which is the accepted potency of this material. When A.T. 10 was fed on a basis of one four-hundredth the potency of vitamin D_2 (group 90), as suggested by the work of Shohl et al., little curative effect was found as measured by the line test.

[1] Drisdol. Winthrop Chemical Company, New York.

[2] Hytakerol. Winthrop Chemical Company, New York.

TABLE 1.
Antirachitic potency of A.T. 10 and vitamin D_2 as measured by the U.S.P. XI rat assay.[1]

ASSAY NO.	MATERIAL ASSAYED	DAILY DOSE FOR 6 DAYS	DEGREE OF HEALING	POTENCY FOUND PER GRAM	FINAL WEIGHT	INORG. Ca/100 CC. OF SERUM	BONE ASH
			line test	*I.U.*	*gm.*	*mg.*	%
Experiment I							
92	U.S.P.[2] reference oil no. 2	6.96 mg.	1.9+	115	69.0		
89	Vitamin D_2[3]	0.02 μg.	2+	40,000,000	75.0		
90	A.T.10	8.0 μg.	0.3+	Dose too small	68.0		
Experiment II							
127	Negative controls	0	0.2+	0	79.0	10.1	34.3
126	U.S.P.[2] reference oil no. 2	6.96 mg.	2.0+	115	75.0	11.8	34.0
121	A.T. 10	13.3 μg.	0.6+	Dose too small	73.0	11.5	37.2
122	A.T. 10	26.6 μg.	2.0+	30,000	76.0	11.6	37.0
Experiment III							
229	Negative controls	0	0	0	71.5	8.2	30.3
147	Positive controls[4]		Complete calcification		146.4	9.0	58.1
228	U.S.P.[2] reference oil no. 2	6.96 mg.	2.0+	115	74.4	10.0	33.7
227	A.T. 10	26.6 μg.	1.9+	30,000	78.8	10.1	34.1
226	A.T. 10	78.0 μg.	3.1+	No toxicity	76.9	10.2	37.3

[1] Values represent pooled samples from eight to ten animals per group.
[2] Standardized, labeled, potency of 115 I.U. per gram.
[3] Expected potency of 40,000,000 I.U. per gram.
[4] Raised on an adequate diet.

Higher levels of A.T. 10 were administered in the second and third experiments. At 13.3 μg. daily (group 121), the compound again failed to show adequate calcification in the epiphysis of the bones. However, when given at a level of 26.6 μg. (groups 122 and 227), a good 2 plus healing was observed, comparable to that obtained from the reference oil (groups 126 and 228). This indicated a vitamin D activity for A.T. 10 of 30,000 I.U. per gram or approximately 1/1300 that of vitamin D_2 as assayed by the U.S.P. XI curative technique.

No toxicity was noted at a higher level fed, 78.0 μg. (group 226); these animals simply demonstrated a greater degree of calcification. The ash content of the bones in all the groups was low when compared with normal rats of that age (group 147), indicating that in this type of assay recalcification of the rachitic bones had not gone very far during the short test period. The amounts of A.T. 10 administered to these rats had no significant effect on raising the serum calcium values above normal.

CHICK EXPERIMENTS AND RESULTS

Single comb White Leghorn chicks were started on assay when 1 day old (weight 30 to 35 gm.) and the experiments terminated on the twenty-first day. Care of the animals, basal diet employed, and details of experimental technique were essentially as described in earlier papers (Correll and Wise, '38; Correll, '41). All values in the tables represent pooled samples from at least fifteen birds for each group.

The oils that were tested were diluted with sesame oil and adjusted to bring the total oil supplement of the feed to 1%. Vitamin D_2 dilutions were made with propylene glycol, the vehicle employed in the commercial preparation used. Again the solutions were calculated to be of such potency that the final test ration would be 1% propylene glycol. In the experiments comparing vitamin D_2 and A.T. 10, all diets including the controls were made identical by the addition of both 1% sesame oil and 1% propylene glycol, with or without the

active principles as the study required. The cod liver oil fed had a potency of 150 I.U. per gram.

The first experiment was planned to compare the response of the chick to A.T. 10 and vitamin D_2 on a weight and unit basis, and to determine the physiologic activity of several levels of A.T. 10 in the birds. In our hands ('38), chicks on this type of experiment require around 25 I.U. of vitamin D from cod liver oil per 100 gm. of diet for minimum protection. Hence, one such group was included to serve as positive controls.

Table 2, experiment I, presents the results of this research. Vitamin D_2, when fed at a level of 2.5 µg., or 100 I.U. per 100 gm. of diet (group 437), gave no protection to the birds, but at a level of 25 µg., 1000 I.U. (group 438), the bone ash values obtained were as high as those of the reference group 432 and the phosphatase values nearly as low. Such a dose of vitamin D_2 approaches closely the minimum requirement. In contrast to this, A.T. 10 fed at 20 µg., 0.6 I.U. per 100 gm. of diet (group 433), or at nearly equal the weight of vitamin D_2 found effective, failed to elicit any antirachitic response. A.T. 10 at 50 µg., 1.5 I.U. (group 440), demonstrated some protection as seen by the bone ash, serum calcium and phosphatase values obtained. When supplemented into 100 gm. of ration in the amount of 200 µg., 6 I.U. (group 434), A.T. 10 afforded complete protection against rickets for the birds. In consideration of the responses obtained from some preliminary experiments and group 440, table 2, this dose of 200 µg. or 6 I.U. appears to be very close to the minimum protective dose for A.T. 10. Thus, on a weight basis, A.T. 10 is about one-eighth as effective for the chick as vitamin D_2. When compared, however, on a rat unit basis, A.T. 10 is nearly 200 times more beneficial than vitamin D_2.

Some indication of toxicity for the chick from A.T. 10 was noted in group 435, both from the appearance of the birds and the lowered final weight. The larger dose fed to group 436 was definitely deleterious. For chickens the toxic dose

TABLE 2

Comparison of the antirachitic effect of A.T. 10, vitamin D_2, and vitamin D from cod liver oil in chicks.

ASSAY NO.	MATERIAL ASSAYED	DOSE PER 100 GM. OF DIET	FINAL AVERAGE WEIGHT	INORG. Ca/100 CC. OF SERUM	INORG. P/100 CC. OF SERUM	PHOSPHATASE PER 100 CC. OF SERUM	BONE ASH	INTERPRETATION
			gm.	*mg.*	*mg.*	*units*	%	
Experiment I								
431	Negative controls	0	123	7.0	6.6	120	38.0	Rachitic
432	Cod liver oil	166 mg. 25 I.U.	142	9.7	7.5	50	46.6	Normal
437	Vitamin D_2	2.5 μg. 100 I.U.	115	7.3	6.7	114	38.3	Rachitic
438	Vitamin D_2	25.0 μg. 1000 I.U.	142	9.5	7.9	62	46.3	Normal
433	A.T. 10	20 μg. 0.6 I.U.	143	7.0	6.6	117	37.7	Rachitic
440	A.T. 10	50 μg. 1.5 I.U.	132	9.1	6.5	75	44.5	Substantial protection
434	A.T. 10	200 μg. 6 I.U.	151	9.8	7.1	49	48.5	Normal
435	A.T. 10	1.0 mg. 30 I.U.	112	11.0	6.7	37	47.1	Slight indication of toxicity
436	A.T. 10	3.0 mg. 90 I.U.	89	11.2	5.9	30	44.9	Toxicity indicated
Experiment II								
441	Negative controls	0	136	4.3	5.5	169	33.6	Rachitic
444	Cod liver oil	20 mg. 3 I.U.	138	5.7	6.1	122	35.5	Slight protection
443	Cod liver oil	40 mg. 6 I.U.	141	5.1	6.2	135	35.2	Slight protection
442	Cod liver oil	166 mg. 25 I.U.	147	8.0	6.9	66	45.4	Normal
447	A.T. 10	50 μg. 1.5 I.U.	140	6.3	6.1	99	38.5	Definite protection
446	A.T. 10	100 μg. 3 I.U.	152	6.7	6.6	103	42.0	Substantial protection
445	A.T. 10	200 μg. 6 I.U.	150	9.1	7.3	47	47.5	Normal

of A.T. 10 is apparently about five times the minimum protective level.

Serum calcium concentration of these chicks with normal parathyroid glands was not significantly altered except where the levels of administered A.T. 10 tended to be morbific. In such groups there was a rise in the calcium values.

As a result of this chick experiment the data revealed an unexpected development. On a rat unit basis, apparently A.T. 10 was even more effective in maintaining high bone ash, low serum phosphatase, and normal serum phosphorus and calcium values, than was the vitamin D from cod liver oil itself. A final investigation was organized to compare more exactly the relative efficacy of these two antirachitics for the chick. In table 2, experiment II, the results of this study are recorded.

In agreement with previous results, vitamin D from cod liver oil at a level of 25 I.U. per 100 gm. of ration (group 442) proved adequate to protect the chicks from rickets. In this particular assay the dose appears to be border line as the serum calcium and bone ash values are a trifle lower than usual and the phosphatase slightly elevated. This only strengthens the comparison and categorically demonstrates that 6 I.U. from A.T. 10 (group 445) is as effective as 25 I.U. from cod liver oil. Further support is lent to this conclusion by comparing the results obtained from 6 I.U. of vitamin D from cod liver oil (group 443), which evidenced but slight protection, with the normal values found when 6 I.U. were given from A.T. 10 (group 445). A.T. 10, at a level of 3 I.U. per 100 gm. of diet (group 446), demonstrated substantial protection while the same dose from cod liver oil (group 444) was only slightly better than the negative controls (group 441). In fact, 1.5 I.U. from A.T. 10 (group 447) was more effective than 6 I.U. from cod liver oil (group 443). Under the conditions of these experiments, rat unit for rat unit, A.T. 10 was four to five times more efficacious as an antirachitic for the chick than was the vitamin D from cod liver oil.

DISCUSSION

There is little published data concerning the antirachitic activity of A.T. 10. The material has been standardized on the basis of its toxicity for mice. Using an entirely different technique than the one employed in this work, Shohl and Farber ('41) concluded that in their experiments A.T. 10 was one four-hundredth as potent as vitamin D_2. No attempt was made to state its antirachitic capacity in units. However, vitamin D_2 has an established strength of 40,000,000 I.U. per gram so those studies which found A.T. 10 to be one four-hundredth as active, circumstantially indicate A.T. 10 to have a potency of 100,000 I.U. per gram on the basis of the protective technique.

No difficulty was experienced in this laboratory in finding a level of A.T. 10 which, when assayed on rats in exact accordance with the U.S.P. procedure for measuring vitamin D activity, would repeatedly yield results comparable to those obtained from the reference oil. At this level A.T. 10 indicated an antirachitic strength of 30,000 I.U. per gram. That is, by the curative method a rat required 26.6 μg. daily of A.T. 10 to show a 2+ healing; 0.02 μg. of vitamin D_2 gave the same response. In the protective method of Shohl and Farber 25 μg. of A.T. 10 prevented rickets and 0.0625 μg. of vitamin D_2 yielded comparable results. Thus with the curative procedure A.T. 10 has one thirteen-hundredths the antirachitic effect of vitamin D_2 as contrasted to the one four-hundredths indicated by preventive feeding. Since the primary purpose of our study was to investigate the possible antirachitic activity of A.T. 10 for the chick, the rat studies were limited to establishing its U.S.P. potency without reference to toxicity.

In a prophylactic type of experiment with chickens, A.T. 10 was found to afford a minimum protective dose when mixed into the ration at a level of approximately 200 μg. or 6 I.U. per 100 gm. of diet. Again detailed toxicity studies were reserved for future investigation and are incidental to this report. However, in feeding the higher amounts of A.T. 10 to the chicks, it was observed that such groups demonstrated lowered

final weights and ragged gross appearance. Using these criteria, a toxic dose was noted at a level of about 1 mg. or 30 I.U. per 100 gm. of diet. Thus the protective dose was one-fifth the toxic dose for chicks and agrees with the findings of Shohl and Farber ('41) who used rats. Vitamin D_2 was not administered at levels high enough to manifest any toxicity; if the response of the chick were again similar to that reported for the rat it should require around 10,000,000 I.U. of vitamin D_2 per 100 gm. of ration to be deleterious.

About 1000 I.U. of vitamin D_2 per 100 gm. of ration served to protect the birds from rickets. It was, therefore, only one one hundred-sixtieth as effective an antirachitic for chicks as A.T. 10. This observation, that vitamin D_2 was so much less efficient than A.T. 10 in preventing rickets in chicks, is strong evidence that the action of A.T. 10 is not due to contamination with vitamin D_2.

A remarkable fact that shows up in these data is that 6 I.U. of A.T. 10 were as beneficial for the chicks as 25 I.U. from cod liver oil. As far as we are aware this is the first report of an antirachitic material of plant origin demonstrating in chickens anywhere near the efficiency of vitamin D from cod liver oil when compared on a rat unit basis. Many substances have been subjected to the dual rat-chick analysis. Some of these from animal origin have proved equally efficient, and a few more efficient for the chick than vitamin D from cod liver oil (Bills, Massengale, Imboden and Hall, '37). In contrast to this, Grab ('36), among others, reported studies on a whole series of plant sterols, not one of which even approached antirachitic substances of animal origin in relative efficacy for the birds.

The picture is further complicated by the realization that the plant sterol A.T. 10, supposedly dihydrotachysterol, is a reduction product of irradiated ergosterol and hence chemically very closely related to vitamin D_2. Yet vitamin D_2 is only about one-fourtieth as effective, rat unit for rat unit, as vitamin D from cod liver oil on the chick, while a rat unit

of A.T. 10 is four or five times more efficacious than one from cod liver oil.

The doses of A.T. 10 fed did not influence serum calcium values so that they differed from those obtained with the reference oils, except in the two groups of chicks which were offered the largest quantities. This is additional evidence that A.T. 10 will only demonstrate an elevation in serum calcium when administered to parathyroidectomized animals; or to normal animals with functioning parathyroids in amounts massive enough to be harmful. It will also be noted that, similar to the action of an antirachitic, increasing quantities of A.T. 10 resulted in decreasing phosphatase values, instead of causing a rise as has been reported for parathyroid hormone. Thus, within the limitations of these experiments the biologic action of A.T. 10 resembled that of vitamin D rather than that of the parathyroid hormone.

SUMMARY

1. A.T. 10 has an antirachitic potency of 30,000 I.U. per gram as assayed by the U.S.P. XI rat technique.

2. A minimum protective level of A.T. 10 against rickets for chicks is around 6 I.U. of antirachitic activity per 100 gm. of ration. A toxic level is apparently about five times this dose.

3. By weight comparison A.T. 10 is approximately one-eighth as potent an antirachitic for chicks as vitamin D_2. However, on a rat unit basis, A.T. 10 is nearly 200 times more effective than vitamin D_2.

4. Rat unit for rat unit, A.T. 10 is about four times more powerful an antirachitic on the chick than vitamin D from cod liver oil. This is the first time an antirachitic substance of supposedly vegetable origin has demonstrated in chickens anywhere near the rickets preventive ability of vitamin D from cod liver oil when compared on a rat unit basis.

5. Under the conditions of these experiments the action of A.T. 10 resembles that of vitamin D rather than parathyroid hormone.

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THE IRON METABOLISM AND REQUIREMENT OF YOUNG WOMEN [1]

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Recent developments in the field of iron research have challenged the long accepted "balance" study as an adequate method for investigating the iron metabolism and requirement of humans. When iron requirements were formulated from such studies it was assumed that the excretion reflects the body's need or daily use of iron and that this need is measured by the difference between the intake and outgo. When interpreted in this way an excretion which equalled the intake indicated that the body's need was just being met, if the excretion exceeded the intake the need was not being met, and if the excretion was exceeded by the intake iron was being stored. It now appears, however, that iron excretion is not a criterion of the body's need or utilization of iron but rather a reflection of the rate or completeness of absorption and that many factors other than need may influence absorption.

McCance and Widdowson ('38) have reported that when 7 mg. of iron daily were given intravenously for 2 weeks to six adults already in iron equilibrium their level of excretion did not rise. They present this observation as the culminating evidence for the new theory that the intestine has no ability to regulate by excretion the amount of iron in the body and that the iron found in the feces is only that which has escaped

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absorption rather than iron which has been absorbed, utilized, and re-excreted.

Hahn et al. ('39) injected five dogs with radioactive iron and from the metabolism results concluded that the iron stores of the body are controlled by absorption or lack of absorption from the intestine rather than by excretion into the intestine.

Further evidence in support of this hypothesis was presented by Leverton ('41) in a study planned to determine whether the fecal excretion of iron would ever significantly exceed the intake, regardless of how low the intake became. Four healthy college girls were kept for several months on a diet that contained only 3.5 mg. of iron daily, but was adequate in other respects. Their average fecal excretion of iron did not exceed the daily intake, thus indicating no elimination of iron through the walls of the intestine.

With iron, unlike other minerals, more than a state of equilibrium is to be desired in young women; there must be storage to replace the iron lost in the menses. This means that regardless of the theories of excretion metabolism results must show positive balances if optimum nutrition in respect to iron is to be maintained.

Barer and Fowler ('36) have found the iron loss during normal menstruation to approximate 20 mg. This would require an average daily storage of 0.7 mg. during a 28-day menstrual cycle to prevent a gradual depletion of body iron. As a recommendation, however, a figure somewhat above the average, at least 1 mg. a day, would be advisable to insure some margin to cover the individual variations in losses greater than 20 mg.

The iron work to be reported here was part of a study of the mineral and nitrogen metabolism of young women planned to determine their self-chosen intakes and subsequent balances of calcium, phosphorus, iron, copper and nitrogen. It was particularly hoped that information would be secured which would clarify the interrelationship of the needs for different nutrients as well as furnish additional bases for dietary recommendations for this age group.

PROCEDURE

Between the fall of 1937 and the spring of 1940 ninety-nine 1-week metabolism studies were made of sixty-nine women students who were between the ages of 16 and 27 years and were living on self-chosen diets. Fifty-two subjects were studied once; seven, twice; seven, three times; and three, four times. The repeat studies were made 6 months to a year apart so that a girl was in a different age group each time she was studied. All of the girls were in normal health as judged by a general medical examination and critical inspection. Studies were made during the inter-menstrual periods. Most of the girls were doing light housekeeping, some were living at home, and a very few ate at the college cafeteria.

A real effort was made to enable the subjects to continue their usual mode of living and dietary habits during the week of study. Each girl was told that the study to be made on her was not one of correct or incorrect dietary practices, but rather of how much of the food she ordinarily ate was stored in her body, and thus she realized that the success of the study depended primarily upon her continuing her usual dietary and living habits. Her accuracy, dependability, and careful cooperation were solicited but she was also encouraged to live in her usual manner as casually as possible.

All of the food eaten was weighed and sampled for analysis and the feces and urine were collected for the period of study. Carmine was given to mark the stools at the beginning and end of the week. The aliquot food samples were combined into a 1-week composite as were the stools and also the daily urine collections. As previously described (Leverton, '41), the separate composites of foods and feces were made into brown digests with 20% hydrochloric acid. Aliquots of the brown digests were oxidized and iron was determined as the thiocyanate. Every care was taken to prevent contamination of the materials during or following collection, and the analytical work was done in a laboratory especially equipped to preclude iron contamination. Calcium, phosphorus, nitrogen, and copper determinations were also made.

RESULTS

Because the greatest value of these results lies in the number of studies and, therefore, the cross-section of metabolic activity that they represent for young women of this age, average figures will be given greater emphasis than individual ones.

The average intake, excretion, and storage of iron for the whole group are given in table 1.

TABLE 1

Intake, excretion, and storage of iron

SOURCE OF DATA	IRON DAILY		
	Intake	Excretion	Storage
	mg.	*mg.*	*mg.*
69 studies of 69 individuals	10.71	9.38	1.33
99 studies of 69 individuals	10.44	9.07	1.37

These average daily intakes of iron permitted a liberal storage for replacement of menstrual loss. The slight drop in intake and excretion values when the data from the repeated studies are included in the averages is probably not significant, especially since the amount of iron that was stored was practically the same in both cases.

In order to analyze differences and still deal with a group of subjects rather than individual cases the studies have been sorted into five groups according to the level of iron intake of the individual cases.

Table 2 contains the average intake and excretion of iron of the subjects in each group accompanied by the average of the heights, weights, and ages of each group. While individual daily intakes of iron range from 5.94 to 16.71 mg., sixty-two of the ninety-nine values fall between 8.00 and 11.99 mg.

In no group did the average of the daily excretions exceed the average of the daily intakes of iron to result in a so-called negative balance. More important than this, however, is the decrease in the percentage of individual cases of negative balances and the steady increase in the amount of iron stored

as the level of intake rose. These two facts indicate that for average individuals in general the long-accepted relation between iron intake and iron absorption still holds. The average storage of the group with the lowest daily intake, 5.94 to 7.99 mg., was not great enough to cover normal menstrual losses, as was the case for the other groups.

TABLE 2

Average of iron metabolism data for ninety-nine studies according to level of iron intake

RANGE OF DAILY INTAKE OF IRON	NO. STUDIES	IRON DAILY			IN NEGATIVE BALANCE	AVERAGE			
		Intake	Excretion	Storage		Ht.	Wt.	Age	
mg.		*mg.*	*mg.*	*mg.*	%	*cm.*	*kg.*	*yr.*	*mo.*
5.94– 7.99	15	7.16	6.75	0.41	40	160.8	53.7	20	10
8.00– 9.99	31	9.18	8.21	0.96	23	162.1	56.0	20	7
10.00–11.99	31	10.90	9.53	1.34	19	164.3	56.8	20	7
12.00–13.99	15	12.91	11.15	1.77	13	164.8	58.8	21	11
14.00–16.71	7	15.85	11.30	4.55	0	163.9	58.2	20	7

Previous publications from this laboratory present evidence that dietary essentials other than iron are important factors in preventing or correcting simple anemia in young women. For this reason in table 3 the diets of all subjects who were storing iron and the diets of all the subjects in negative balance are compared with respect to iron, calcium, phosphorus, and nitrogen. While these nutrients are not the only ones by which to judge the adequacy of the diets they are recognized as important objective criteria.

The consistency with which the differences are always in favor of the group that is storing iron is probably of greater significance than are the actual numerical differences between content of the diets of the two groups.

These numerical differences are greatly accentuated, however, at levels of iron intake below 8 mg. This is also shown in table 3 when the fifteen individual studies in which the daily iron intakes ranged from 5.94 to 7.99 mg. are similarly divided into those storing iron and those not storing it.

These figures of iron intake and balance show the limitations to the value of average figures. In table 2 the group with this low intake, 5.94 to 7.99 mg., did not have a storage sufficient to cover menstrual losses but here the figures show that those who were storing iron did so to a greater extent than 1.37 mg. which was the average for all ninety-nine studies.

TABLE 3

Comparison of intakes of iron, calcium, phosphorus, and nitrogen

SOURCE OF DATA	AVERAGE DAILY					HEMOGLOBIN
	Iron		Ca	P	N	
	Intake	Balance	Intake			
	mg.	*mg.*	*gm.*	*gm.*	*gm.*	*gm./100 ml.*
For entire group:						
78 studies of 52 individuals storing iron	10.75	+2.11	0.88	1.36	9.70	13.3
21 studies of 17 individuals not storing iron	9.29	—0.82	0.77	1.13	8.64	12.8
For subjects with daily iron intakes below 8 mg.:						
9 studies of 7 individuals storing iron	7.21	+1.55	1.04	1.27	9.70	13.3
6 studies of 5 individuals not storing iron	7.09	—1.30	0.66	0.94	7.25	12.0

It would appear then from the differences in the calcium, phosphorus, and nitrogen content of the diets that when the daily iron intake of young women is below 8 mg. the adequacy of the diet in other nutrients, probably not just those discussed here, is the deciding factor in stimulating storage for replacement of that lost in menstruation and perhaps for other functions not so clearly understood or measurable. Several years of contact with the girls, and their families if the girls lived at home, leave no doubt in the minds of the authors as to the general superiority of the food habits and nutritional history of the seven girls who were storing iron at this low level of intake as compared with the five who were not.

This is specifically illustrated by the two cases shown in table 4 — subject 69 and subject 17 who were living at home and eating meals prepared by their mothers. Subject 69 had an unquestionably good dietary history and good food habits; although she cared little for meat she ate abundantly of milk and cheese, fruits and vegetables and used whole wheat bread and cereals. Subject 17 was not conscious of having poor food habits but had practically never drunk milk, did not care for vegetables and ate white bread entirely.

TABLE 4

Comparison of subjects 69 and 17

SUBJECT	AGE WHEN STUDIED		AVERAGE DAILY				
			Iron		Calcium intake	Nitrogen	
			Intake	Balance		Total	Per kilo
	yr.	*mo.*	*mg.*	*mg.*	*gm.*	*gm.*	*gm.*
No. 69 on the better diet	17	1	7.47	+3.47	1.08	9.28	0.180
	17	8	7.41	+1.75	1.17	11.28	0.221
	18	2	5.94	+0.77	1.08	9.32	0.182
No. 17 on the poorer diet	19	7	7.89	—1.65	0.53	5.89	0.090
	20	3	6.29	—1.02	0.44	6.95	0.116

DISCUSSION

If one accepts the premise that the intestinal tract does not re-excrete iron the significance of observed negative balances in iron metabolism studies is problematic. Their occurrence may be attributable to such factors as: (1) too short a period of study; (2) an atypical state of the subject; (3) difficulty in separating the carmine-marked and unmarked portions of the feces; and (4) an abnormal permeability of the individual's intestinal tract to the re-excretion of iron from the body. The same factors together with adsorption of iron by the intestinal mucosa could also operate to produce fictitious storages of iron. While these factors may apply to occasional individual cases it may be safely assumed that ninety-nine studies would give a typical cross-section of the iron metabolism of a group

of normal young women and show whether they were storing enough of this element to replace menstrual losses.

The practical significance of these results is the finding that the customary intake of iron of young women on self-chosen diets permits a storage of more than enough to replace the iron lost in the menses and that two-thirds of this amount would be sufficient if the diet were generous in other nutrients.

The figure of 10.44 mg., representing the average daily intake of the group and associated with a daily storage of 1.37 mg., is valuable in showing group performance but it overlooks the individual subjects who were in negative balance. In these cases of apparent negative balance, regardless of the level of intake, the correction would seem to be in the general improvement of the diet with larger quantities of protective foods rather than by an increased intake of iron alone.

This influence of the quality of the diet upon the absorption of iron, especially at low levels of intake, has been observed in other studies in this laboratory. When a group of college women with simple hypochromic anemia was given daily supplements of vitamin B complex or additional protein from dry milk, cheese, and peanuts the hemoglobin content of their blood increased even though no medicinal iron was given (Leverton and Marsh, '41). Also when four young women had a generous diet except that the daily iron intakes were between 3.5 and 4.5 mg. for several months they remained in excellent health and there was no great decrease in hemoglobin (Leverton, '41). When their iron intake was increased to 6.55 mg. their average daily storage was 2.44 mg.

The bearing of these findings upon the dietary recommendations for iron is less than might be expected. While the results show that the chances of storing iron are greater at higher levels of intake they also show that ample storage occurred at surprisingly low intakes when the diet was rich in other nutrients. A person's intake could be high in iron and poor in protective foods but if it were rich in protective foods it could hardly be deficient in iron — both because the protective

foods are good sources of iron and because the amount of iron required appears to be small if the supply of other nutrients is liberal. It follows then that a well-balanced optimum diet probably holds more promise than does iron medication for a permanent general improvement in the treatment of simple hypochromic anemia in young women.

SUMMARY AND CONCLUSIONS

Ninety-nine 1-week iron metabolism studies were made on sixty-nine young women between the ages of 16 and 27 years who were living on self-chosen diets.

The average daily intake of 10.44 mg. of iron provided an average daily storage of 1.37 mg. which is ample to replace normal menstrual losses. As the level of intake rose from below 8 mg. to 16 mg. the occurrence of negative balances decreased and the amount of iron that was stored increased.

Seven subjects whose diets were generous in other nutrients had an average daily iron intake of 7.21 mg. and were storing 1.55 mg. daily or more than enough iron to replace that lost in normal menstruation. Five other subjects with similar iron intakes but suboptimum diets in other respects had an average daily negative balance of 1.30 mg. of iron.

There is no occasion for suggesting more or less iron than is recommended in current dietary standards for young women. However, calculations to determine whether a diet contains an adequate amount of iron may eventually be discontinued since it appears that emphasis should rather be placed upon obtaining diets optimum in other essential nutrients which have been shown to function in efficient iron absorption and utilization.

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THE EFFECT OF ENZYMATIC DIGESTION ON THE PANTOTHENIC ACID CONTENT OF MEATS DETERMINED BY THE MICRO-BIOLOGICAL METHOD[1]

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In a previous publication (Waisman et al., '39), we reported the relative pantothenic acid potency of animal tissues as determined by bioassay with chicks maintained on a dermatitis producing ration. While these results furnished an approximation of the pantothenic acid in meat products the availability of a rapid microbiological method together with pure calcium pantothenate enabled us to determine the pantothenic acid content of many of the tissues previously assayed on a more quantitative basis. A large number of additional meat samples have also been assayed by this method.

Several microbiological methods have been proposed for the estimation of pantothenic acid. Williams and Saunders ('34) used the yeast growth method, while Pennington, Snell and Williams ('40) and Strong, Feeney and Earle ('41) used the organism Lactobacillus casei ε, and Pelczar and Porter ('41) used Morgan's bacillus.

METHOD

The organism and medium which were employed in this study were those described by Strong, Feeney and Earle ('41).

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Preliminary trials using their method of hot water extraction on animal tissues gave erratic results and it was found that homogenization of the sample in the Potter-Elvehjem ('36) homogenizer followed by the hot water extraction (autoclaving at the natural pH) gave more consistent results. However, the values obtained by this method were not entirely satisfactory. It appeared that an enzymatic digestion would make the pantothenic acid more readily available to the test organism. Although Pennington, Snell and Williams ('40) suggested the use of autolysis of the fresh tissue, we believed that a more thorough digest of the dried samples which we had at our disposal would be advisable.

Since the samples we used contained high amounts of protein, it seemed advisable to try pepsin for the digestion. The pepsin digestions were performed on both fresh and dried samples of the same tissue together with autolysis of fresh tissue at the natural pH. All samples were homogenized before being subjected to the particular extraction procedure. The pantothenic acid values obtained after these treatments are given in table 1. It will be seen that pepsin digestion of either the dried or fresh tissue gave higher results than the untreated fresh or dried tissue. The autolysis of the fresh tissues also produced an increased pantothenic acid value over that for the untreated fresh tissues in all the samples tested.

Further work on enzymatic digestion was carried out using clarase.[2] Several levels of clarase were used with the homogenized suspensions of the dried meat sample. Equal weights of clarase and meat were used as well as twice as much enzyme as meat, and in both cases the pantothenic acid titer was the same. However, the values obtained in all cases were higher than those after pepsin digestion.

The use of a pancreatic extract appeared to us to be a more desirable enzyme preparation to use because of its ability to give more complete liquefaction of the tissue. Several

[2] Obtained from Takamine Laboratories, 193 Arlington Avenue, Passaic, New Jersey.

trials with pancreatin,[3] as well as with clarase and pepsin, on pure calcium pantothenate satisfied us that the enzymes had no effect on the amide linkage of the vitamin and that the quantity of enzyme added for digestion furnished only in-

TABLE 1

Effect of enzymatic digestion and drying procedure on microbiologically determined pantothenic acid.

SAMPLE	MICROGRAMS PANTOTHENIC ACID PER GRAM						
	Fresh [1]				Dry [1]		
	Untreated	Autolyzed	Pepsin digested	Pancreatic digested	Untreated	Pepsin digested	Pancreatic digested
Veal heart 155	120	158	160		125	166	
Pork loin 156	25	30	33		27	35	
Beef round 157	13	16	19		13	21	
Veal liver 158	130	160	159		145	145	
Pork liver 159	159	193	177		145	194	
Pork loin 165				42			45
Beef heart 166				119			122
Beef kidney 167				242			223
Beef liver 168				290			283

[1] Expressed as micrograms calcium pantothenate calculated to the dry weight basis.

significant amounts of pantothenic acid. All the values were corrected by subtracting the amount of pantothenic acid in the enzyme preparation. The pancreatic digests were made in a buffered solution at pH 7.0 to 7.5 so that no pantothenic acid would be destroyed by either acid or alkali.

[3] Merck and Company, Rahway, New Jersey.

The marked increases in apparent pantothenic acid content after enzymatic digestion led to a consideration of the possibility that enzymatic treatment of the tissue liberated some bacterial growth-stimulating substance which accounted for the higher values obtained. After several tests involving numerous recovery experiments we were convinced that the higher results obtained after pancreatic digestion were actually due to increased liberation of pantothenic acid from its natural complexes.

Having assured ourselves that more consistent results could be obtained by enzymatic digestion, we determined whether the use of our dried tissues was satisfactory for the microbiological assays. Accordingly several samples of meat which were representative of both high and low pantothenic acid containing tissues were obtained from a local market. Part of each sample was assayed in both the fresh and dry state. After determining the moisture content of the fresh tissue it was possible to compare the amount of pantothenic acid in both fresh and dried portions on the same basis. As can be seen from the figures under "fresh" and "dry" in table 1, it was apparent that our drying procedure had no deleterious effect on the pantothenic acid content. The vitamin content of the samples tested in the dry state was equal to that of the fresh weight when the pantothenic acid content was expressed in micrograms per gram of the dried tissue.

The pepsin, clarase and pancreatic digestions were performed on the majority of our meat samples, but to conserve space only typical figures are given in table 2. It will be seen that greater amounts of pantothenic acid were obtained after clarase treatment than after pepsin digestion and a still further increase was obtained after pancreatic digestion. Although there were several samples which did not show this progressive increase after treatment with the respective enzymes, the majority of the muscular tissues did give the highest pantothenic acid values after pancreatic digestion. At present, we are satisfied that the most reliable pantothenic acid

figures for animal tissues can be obtained after pancreatic digestion of the homogenized tissue.

The exact procedure used in the microbiological assay of animal tissues using the pancreatic digestion is as follows: A half gram of the finely pulverized or homogenized sample is placed in a suitable brown bottle with a solution containing 50 mg. of pancreatin and 0.5 gm. of K_2HPO_4 and the volume

TABLE 2

Typical pantothenic acid values obtained after various enzymatic digestions on the dried meat.

SAMPLE	SAMPLE NO.	TREATMENT OF MEAT	DIGESTION WITH		
			Pepsin	Clarase	Pancreatin
			Micrograms per gram dried tissue[1]		
Beef liver	98	——	138	152	150
Beef liver	150	——	240	248	240
Veal liver	158	——	145	145	158
Pork liver	153	——	230	221	221
Beef kidney	81	——	158	178	173
Beef round	154	——	38	40	49
Pork ham	139	——	28	32	45
Pork ham	141	Fried	10	19	20
Pork ham	142	Roasted	7	14	27
Pork ham	117	Tenderized	16	32	40
Pork ham	102	Smoked	30	37	44
Pork loin	93	Fried	8	15	22
Beef tongue	82	——	34	—	74

[1] Expressed as calcium pantothenate.

adjusted to 100 ml. with distilled water. The mixture is incubated for 24–72 hours at 38° C. with 1 ml. of toluene added as a preservative. After digestion a suitable aliquot is removed and diluted so that the pantothenic acid content falls in the range required by the assay method. The medium, culture, inoculation, and titration procedures of the assay are essentially those reported by Strong, Feeney and Earle ('41). Table 3 includes the pantothenic acid values for more than eighty samples of animal tissue.

TABLE 3

Microbiologically determined pantothenic acid of pancreatic digested animal tissues.

TISSUE		MICROGRAMS PER GRAM[1]	
		Dry	Fresh
Beef liver	98	150	—
	131	254	74
	150	240	66
	163	208	63
	168	283	88
	152	148	44
Fried	121	103	44
Veal liver	97	262	76
	70	226	60
	158	158	43
Pork liver	159	196	55
	153	221	69
	122	124	39
Lamb liver	96	238	69
Beef kidney	126	160	32
	81	173	40
	167	223	49
Stewed	112	114	40
Pork kidney	83	177	39
	62	127	27
	137	157	33
Beef heart	143	93	18
	133	121	23
	54	56	12
	166	122	22
Stewed	87	63	19
Veal heart	155	121	25
Pork heart	104	114	25
Pork loin	36	63	20
	74	45	13
	125	68	19
	156	69	21
	162	46	14
	165	45	13
Broiled	37	26	6
Fried	38	41	10
,,	93	22	7
Fresh frozen	89	51	17
Veal hindquarter	103	53	14
	134	44	10
	129	72	18
Fried	45	39	—
Lamb leg	80	50	13
Beef spleen	130	63	13
	76	48	—
Stewed	88	42	—
Beef round	105	46	13
	146	51	13
	151	36	—
	154	49	12
	128	29	7
	42	36	10
	157	37	10
	164	28	8
Fried	106	30	—
Broiled	147	34	—
Fried	148	36	—
Pork ham	95	76	19
	32	69	19
	139	45	11
	124	70	17
Fried	141	20	10
,,	52	46	—
Roasted	142	27	13
Tenderized	72	21	7
,,	119	27	9
,,	117	40	14
Smoked	116	58	17
,,	102	67	19
,,	47	44	14
Smoked, fried	48	44	21
Boiled	115	13	5
Beef pancreas	113	106	28
	64	60	19
Beef brain	144	170	36
Beef lung	78	54	—
	138	72	15
Beef tongue	82	74	20
Light muscle of chicken	65	35	9
Light muscle of chicken	68	27	7
Dark muscle of chicken	66	83	21
Filet of cod	135	21	4
Salmon muscle	136	65	18

[1] Expressed as calcium pantothenate.

DISCUSSION

A critical comparison of the values presented in tables 1 and 2 offers several noteworthy observations. The effect of enzymatic digestion on the pantothenic acid values for liver or kidney is evidently much less than that obtained on muscular tissue. No marked increases in the pantothenic acid values of liver and kidney were observed after pancreatic digestion when compared to the values after clarase and pepsin digestion. Clarase digestion of beef, veal, pork and lamb muscle tissue caused a very definite increase in pantothenic acid content over the values obtained after pepsin digestion of these tissues. In general there was a still further increase in the pantothenic acid content of muscle tissue when the samples were treated with pancreatin. In several samples of heart muscle there was an indication of increased pantothenic acid content both with clarase and pancreatin treatment. The same results were obtained with beef tongue indicating a definite trend toward greater liberation of pantothenic acid in those tissues which contained increased amounts of muscular fiber. However, a sample of beef brain showed more than double the pantothenic acid content after pancreatic digestion when compared to the amount found after pepsin digestion.

The variable effect of the different enzymes on certain of the tissues may furnish some clue as to the form in which pantothenic acid occurs within animal tissues. There are at least two explanations for the apparently greater quantity of "free pantothenic acid" in liver as contrasted with other animal tissues. It would appear likely that in a more highly metabolizing tissue as the liver and kidney, the pantothenic acid occurs free to a greater extent than in structural tissue such as muscle. A second explanation is the probable occurrence of a larger number of enzymes in the liver than in other organs or muscle which would allow increased autolysis. The greater "apparent pantothenic acid" content would thus actually be due to increased amounts liberated from the natural pantothenic acid complexes during preparation. This second explanation finds support in the report by Rohrman

et al. ('34) that fresh liver contains a relatively low pantothenic acid content which increases twelve times upon autolysis. Our drying procedure evidently allowed sufficient autolysis to occur since hot water extraction removed the greater proportion of pantothenic acid in the liver samples. While pepsin digestion gave a marked increase in pantothenic acid content of muscle over that given by simple water extraction, it is probable that a more complete breakdown with pancreatin would give greater liberation of the combined pantothenic acid. Although it would appear that the bacteria could utilize all the free pantothenic acid in the meat preparation, the possibility does exist that the vitamin may be mechanically held within the particles of meat and thus be unavailable to the organism. Any attempts to obtain more complete solution of the tissue would minimize this possibility.

It will be seen that the greatest increases in pantothenic acid were found after pancreatic digestion of cooked meats. For example the sample of stewed heart showed a threefold increase in pantothenic acid content after pancreatic digestion when compared to pepsin digestion. Stewed beef spleen showed twice the vitamin content and several of the commercially treated pork muscle samples showed a two- and threefold increase in vitamin content after the more complete hydrolysis of the sample. The denaturation of the meat proteins may have made the pantothenic acid less extractable after cooking and it was only after pancreatic digestion that the vitamin was completely liberated.

In general, the same relative pantothenic acid potency of the tissues has been found by the microbiological test as by the chick assays. Liver, kidney and heart are outstanding sources of pantothenic acid while muscular and organ tissues such as pancreas, brain, lung, etc., are good sources of the vitamin. The different samples of liver showed some variation in pantothenic acid content but this may be ascribed to the various factors which influence the vitamin content of the carcass such as age of the animal, type of feed consumed, etc.

Such variations within a limited range are also seen in the pantothenic acid values of the muscle tissue.

The loss of pantothenic acid due to household and commercial processing varied somewhat with the type of cooking procedure and with a particular tissue. The pantothenic acid values of stewed beef kidney, stewed beef spleen and stewed beef heart were slightly lower than those found in untreated samples of these tissues. The decrease can be accounted for in large part by the solution of the vitamin in the cooking water. The effect of frying is best shown by comparing parallel samples of fried beef round and untreated beef round from the same cut. Two samples of beef round showed a loss of approximately 30% after frying. Nearly the same loss was observed in the fried pork muscle, but no apparent loss was observed in the fried smoked ham. Several roasted samples showed losses approximating 40%.

Tests on the effect of commercial processing on the stability of pantothenic acid were restricted to pork muscle samples. The tenderizing process caused a loss of 50% of the pantothenic acid while the boiled ham samples showed approximately one-third the original pantothenic acid of the majority of untreated ham samples. Smoking the hams resulted in the least loss of pantothenic acid.

A summary of the vitamin content of meat indicates that liver and kidney are the outstanding sources of nicotinic acid and riboflavin (Waisman and Elvehjem, '41). The results of the pantothenic acid assays showed that liver and kidney also contain high amounts of this vitamin. The muscle tissue and organ tissue are less potent than kidney or liver, yet contain appreciable amounts of pantothenic acid.

SUMMARY

Microbiological assays for pantothenic acid have shown that animal tissues contain considerable amounts of this vitamin. Liver and kidney of the various species were the richest of any of the tissues studied while striated muscle, heart, lung, pancreas, brain and spleen contained appreciable quantities.

It appears that the pantothenic acid in animal tissues occurs in combined form and is liberated most completely by pancreatin digestion. Partial liberation of the vitamin from its complexes was obtained with other enzymes which hydrolyzed the meat proteins less completely. There is a decrease of approximately 30 to 40% of the vitamin in the cooked or commercially processed samples.

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EFFECT OF PECTIN ON THE RETENTION OF DIETARY LEAD (RADIUM D)

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Shields, Mitchell and Ruth ('39) found that inclusion of 32% apple powder in rat diets decreased lead retention by 37% when the food contained 33 p.p.m. of lead as lead arsenate. They point out the insolubility of lead pectate, and suggest that the pectin of the apple may be a principal factor in reducing retention by decreasing the absorbability of this metal. A protective effect of raw apple was reported by Manville et al. ('40), who used even larger amounts of lead arsenate. These authors, however, were able to demonstrate only a better growth of the apple-fed groups (rats, guinea pigs, rabbits). They did not make analyses for lead in the whole animals, but found that bone, kidney, and liver taken from the animals receiving apple might show either more or less lead than the controls.

In order to determine whether pectin itself can decrease the retention of lead, we have carried out experiments on lead retention using purified pectin. We also wished to have the conditions adverse to a demonstration of the possible effect of pectin, and have therefore used a diet high in calcium and with the addition of minimal amounts of lead. Tompsett ('39) presents data which show that the proportion of lead absorbed from the diet decreases with increasing amounts of lead consumed; this effect is most marked on a high calcium diet but is definite on low calcium diets also. The decrease in lead absorption produced by high levels of calcium is greater with

increasing amounts of dietary lead. This effect of calcium on lead absorption has been confirmed by Lederer and Bing ('40). We might therefore expect that other agents which inhibit lead absorption would be less effective when the amount of lead in the diet was in the lower range. By the use of the naturally occurring radioactive lead isotope, radium D, it has been possible to study the effect of pectin on lead absorption when the amounts of lead in the diet are no greater than those present as natural contaminants, since the quantity of radioactive lead which must be added to the diet is negligible in comparison with that already present in most foods. The use of radium D has also made it possible to trace the lead actually absorbed, retained, and excreted, bearing in mind the fact that none of the lead determined by the method could have been present previously in the body of the animal.

PROCEDURE

Radium D (Pb^{210}), which has a half period of 16.5 years, was obtained from disintegrated Radon tubes. The tubes were smashed and the mercury they contained, due to the pumping operation, was removed by vacuum distillation in a micro still, using a fine air stream to carry off the vapors. The active deposit was taken up in a mixture containing 1 part concentrated HNO_3, 1 part concentrated HCl, and 2 parts water. It was then evaporated to dryness, taken up in 1:1 concentrated HNO_3, and diluted so that 25 ml. contained 5 ml. of concentrated HNO_3. This solution also contained traces of the short-lived isotopes of bismuth and polonium, which emit chiefly alpha and beta particles with small amounts of soft gamma rays. It was not necessary to remove these from solution, since their radiation can be easily eliminated by screening the Geiger tube. Various dilutions of this Pb^{210} solution in 1:4 HNO_3 were retained as permanent standards.

A known volume of the Pb^{210} solution was added to 300 gm. of dry starch, the mass vacuum dried, transferred quantitatively to a ball mill, and powdered for 24 hours. Portions of the lead-starch mixture were analysed for radioactivity in

comparison with the permanent standards, and were found to check within 2% of the calculated values. This lead-starch preparation was used in place of a part of the ordinary starch in the diet, the amount added being calculated to supply 1150 "units" of activity per 200 gm. of diet.

The difficulty of determining the weight of lead added to the diet when the only known figure is the approximate radioactivity of the original Radon tubes, is obvious. However, it may be roughly estimated that the amount of lead fed to each rat could not have been more than 8 µg., i.e., the diet would contain not more than 0.04 p.p.m. of radioactive lead. Although the diet was not analysed for total lead, it may be assumed from the data of other investigators on the lead content of common foods that the radioactive lead amounted to only a small fraction of the total lead in the diet. It is certain that the total lead content of the diet was not appreciably higher than that of the usual rat diet.

Four litter mate pairs of rats were used, of which half received the control diet and the others the same diet but with 5% of the ordinary starch replaced by pectin. The compositions of the diets are given in table 1.

TABLE 1

Composition of control and pectin-containing diets.

	CONTROL DIET	PECTIN DIET
	%	%
Whole milk powder	50	50
Starch [1]	39	34
Purified casein	5	5
Dried brewers' yeast	4	4
Sodium chloride	1	1
Osborne-Mendel salt mixture	1	1
Purified pectin [2]	0	5

[1] Consisted of a mixture of ordinary starch with starch to which Pb^{210} had been added, the amount of Pb^{210} starch being the same in each diet.

[2] Citrus products 194 grade, slow set. This was purified by four washings with strongly acidified 60% alcohol followed by one washing with 95% alcohol and air drying.

The animals were kept in all-glass cages and fed from porcelain cups. Urine and feces were caught in glass funnels provided with glass wool for screening and the urine was retained in small flasks. The diets were restricted to prevent large gains in weight, and were mixed with water in the feeding cups to avoid wasting and contamination of the excreta. Each animal was fed 200 gm. of diet which contained 1150 "units" of radioactivity. When this amount had been consumed the animal was etherized, and fecal matter removed from the gastrointestinal tract. Analyses for radioactivity were then carried out separately on the urine and feces for the entire experimental period and on the whole animal.

Preparation of samples for analyses. The usual dry and wet ashing procedures were found tedious and difficult when applied to these samples. We have therefore employed a nitric acid isobutyl alcohol digestion which is simpler and less subject to error. The feces were collected and combined with the contents of the intestine. The glass rat cage was rinsed with 1:1 nitric acid and then with 10% acetic acid and the washings added to the urine sample. The urine, feces, and rat carcass were each placed in a 3 liter beaker and covered with concentrated nitric acid. After frothing had subsided a few milliliters of isobutyl alcohol were added very cautiously while stirring. The violent reaction that results serves to bring the samples into solution very rapidly. In the case of the rat carcass, the fat rises to the surface upon cooling, and is removed with hexane in a separatory funnel, the hexane being washed with cold water. Removal of the fatty acids in this manner aids in further handling of the sample.

The beakers were then placed on asbestos covered low heat hot plates and allowed to evaporate nearly to dryness. The samples were transferred with hot acidified (HNO_3) water to porcelain evaporating dishes, evaporated to dryness on the steam bath and dehydrated in the oven, and finally ashed slowly in the furnace without ignition and below 500° C. With the preliminary treatment, ashing became simple and could easily be accomplished in a few minutes without loss of mate-

rial. About 20 ml. of concentrated HCl were added to the ash and evaporated to dryness on the steam bath. The residue was taken up in hot hydrochloric-citric acid solution containing 100 gm. of citric acid and 100 ml. concentrated HCl per liter, and filtered through a fine sintered glass filter. The filter and evaporating dish were washed successively with hot HCl, hot HCl citric acid solution, and hot 40% ammonium acetate. A few pellets of NaOH were then added to the evaporating dish, dissolved in a few milliliters of water with rotation to insure complete wetting of the dish, and this solution evaporated to dryness. The residue was taken up with hot water and this was poured directly into the filtrate. The dish was finally rinsed with hot 1:4 HCl. The combined filtrate and washings were cooled, 20 ml. of 50% citric acid solution were added, and the pH was adjusted to 3.0–3.4 with concentrated ammonia using bromophenol blue as an outside indicator. Five milligrams each of copper sulfate and lead nitrate were added to the solution, and the heavy metals were precipitated by a stream of H_2S. The solution was filtered immediately on a fine sintered glass filter, and the sulfides remaining in the beaker and on the filter were dissolved with 5 ml. of hot concentrated nitric acid. The nitric acid filtrate containing the dissolved lead was caught in a 25 ml. volumetric flask, cooled, and made to volume. Samples of 5 ml. of the solutions thus prepared were placed in flat bottomed 1.5×4 cm. vials, and sealed. The extra lead and copper added before saturation with H_2S give an easily workable precipitate and insure that the traces of radioactive lead will be quantitatively recovered.

For the determination of radioactivity a Geiger-Mueller apparatus was constructed using the quenching circuit described by Johnson ('38). This was modified to include both the grid potential control as suggested by Coven ('39) and the recording circuit of Neher ('39). The regulated high voltage supply was adapted from that used by Lifschutz ('39). The Geiger-Mueller tube was mounted in a bakelite box with a hole drilled above the tube so that vials containing the radioactive solutions could be placed in exactly the same position

for each determination. By keeping the position of vial and volume of solution constant, the solid angle was the same for each determination. The procedure was such that the concentration of reagents was the same in all of the unknown samples and in the standard Pb^{210} solutions, thus controlling the absorption factor. Counts on the unknown solutions were made alternately with counts on the two standard solutions most nearly equal to the unknown in activity. The average of four 1-hour counts on the unknown was used, and was converted into milliliters of original Pb^{210} solution from a curve plotted with milliliters of original solution as the abscissa and counts per minute as the ordinate.

RESULTS AND DISCUSSION

Table 2 compares the lead retained and excreted via urine and feces in the four litter mate pairs with and without pectin. Since by the procedure used all of the lead should be recoverable, and since contamination with ordinary lead is of no consequence, it has been possible to obtain exceptionally good recoveries of the dietary lead.

The retention of dietary lead, which for our control group averaged 15.8%, may be compared with retentions observed by others who have administered larger amounts. Thus Tompsett ('39) found 28% retained by mice given 0.7 mg. over a 14-day period, when the animals received a high calcium diet. Shields, Mitchell, and Ruth ('39), who fed much larger amounts over longer periods, report 1.0 to 1.5% retention. Kehoe et al. ('40) state that retention is negligible in man when somewhat more than 1 mg. per day is taken over a 3½ year period. The proportion of lead retained is evidently in inverse proportion to both dosage and length of time over which a given daily dosage is administered. The retention of about 16% in our experiments is in line with previous observations from short term, low dosage experiments.

The distribution between urine and feces, as determined by us, is probably somewhat inaccurate since under the conditions of our experiments some leaching of the feces by urine

TABLE 2

Effect of pectin on the retention and excretion of lead (radium D) in the diet of the rat.

DATE GIVEN	INITIAL WEIGHT	FINAL WEIGHT	TOTAL LEAD UNITS FED	TOTAL LEAD UNITS RECOVERED			PER CENT RECOVERED	PERCENTAGE DISTRIBUTION		
				Animal	Urine	Feces		Animal	Urine	Feces
	gm.	*gm.*								
1 Control	152	151	1150	175	120	850	99.5	15.2	10.4	74.0
2 Pectin	153	148	1150	150	87	925	101.5	13.0	7.6	80.4
3 Control	142	159	1150	187	112	825	97.7	16.2	9.7	71.6
4 Pectin	140	148	1150	127	87	913	98.0	10.0	7.6	79.4
5 Control	112	140	1150	185	150	800	98.5	16.1	13.0	69.5
6 Pectin	112	142	1150	154	112	900	101.5	13.4	9.7	78.2
7 Control	152	156	1150	180	122	825	98.0	15.6	10.6	71.7
8 Pectin	152	154	1150	125	75	935	98.5	10.9	6.5	81.2
Means of controls								15.8	10.9	71.7
Means of pectin-fed								11.8	7.9	79.8
Differences of means and standard errors of differences								4.0 ± 0.84	3.1 ± 0.97	8.1 ± 1.11
							t	4.8	3.1	7.3
							P	0.01	0.02	0.001

Statistical analysis of percentage distribution by small sample method (Fisher). Note that figures in per cent can be treated as absolute values, since they are parts of the constant amount fed to each rat.

could have occurred. The 72% of the Pb^{210} eliminated in the feces of the control group is therefore a minimum figure, and the urine values may be slightly too high.

Our results reveal a clear-cut and statistically significant effect of pectin on lead absorption and retention. The amount of lead retained in the body at the end of the experiment is, on the average, 24% less in the pectin-fed rats than in the controls. The difference between the average body lead in control and pectin rats is 4.0 and its standard error ± 0.84. The probability (Fisher's table of t) of this difference occurring by chance is less than 1 in 100. The smaller urinary output of lead and the greater fecal excretion in the pectin-fed animals as compared with the controls are likewise statistically significant, P being 0.02 and 0.001 respectively. In order to interpret the greater fecal excretion and smaller body and urine content of the pectin-fed animals as evidence of diminished absorption, it is of course necessary to neglect the possibility of absorption and re-excretion via the digestive tract. However, we know of no reason why the administration of pectin should increase biliary or intestinal lead secretion. On the other hand, an inhibition of lead absorption by pectin is readily understandable. Not only pectic acid, but pectin itself forms insoluble lead compounds. Kertesz ('40) has shown that pectin passes unchanged through the small intestine, and we have confirmed his findings in this laboratory (unpublished). The formation of an insoluble lead-pectin complex would therefore be expected to result in the transmission of increased amounts of lead through the small bowel, and although pectin may be broken down in the large intestine by bacterial fermentation there would be relatively little absorption of the liberated lead in this region. The addition of 5% pectin to the diet does not cause diarrhea and we have no reason to believe that the diminished absorption of lead can be accounted for by an increased rate of passage through the small intestine.

Finally, it should be emphasized (1) that the total amount of lead in the diet used by us is that which may be found in the average diet of rat or man, (2) that the diet was rich in

calcium, and (3) that if the effect of pectin on lead absorption is analogous to that of calcium one would anticipate more marked effects with increasing concentrations of dietary lead. This indicates that the 24% reduction in lead retention which we have observed is likely to be exceeded under less unfavorable conditions.

SUMMARY

Rats were fed 200 gm. each of a diet containing not more than 0.04 p.p.m. of Pb^{210}. One group served as controls, another received 5% pectin which replaced an equal amount of starch.

The control group retained 15.8% of the radioactive lead, and excreted 10.9% in the urine and 71.7% in the feces. The pectin-fed animals retained an average of 24% less lead than the controls. Significantly less was eliminated in the urine and significantly more in the feces.

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VITAMIN SUPPLEMENTATION OF U. S. ARMY RATIONS IN RELATION TO FATIGUE AND THE ABILITY TO DO MUSCULAR WORK [1]

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The recognition of borderline vitamin deficiency states prompts the suggestion that a direct relation between the intake of certain vitamins and physical vigor may extend even above the subclinical deficiencies. During the past several years the popular belief has grown that a superabundance of these vitamins may produce extraordinary stamina or at least may be helpful in meeting physical or mental strain.

These possibilities were of such obvious importance as to merit careful investigation. This paper is a report on such studies made under rigidly controlled conditions on personnel of the U. S. Army.

EXPERIMENTAL PROGRAM

The general plan was to study the physiological and biochemical responses to standard exercise in representative volunteers. Four main experimental series were carried out on twenty-six soldiers, involving a total of 256 experiments.

[1] This work was sponsored by the National Research Council, Division of Medical Sciences and was specifically approved by the Subcommittee on Clinical Investigation and the Subcommittee on Nutrition. The use of soldiers as subjects was authorized by the Secretary of War, acting on the recommendation of the Surgeon-General, U. S. Army. Special financial assistance was also provided by the Mayo Foundation and by the Department of Athletics, University of Minnesota. Important aid was rendered by the Works Progress Administration under the University of Minnesota Project No. 8760, Sub-project No. 380.

Throughout the entire period of study all the men subsisted on standard Army Post rations; during part of this time they received daily supplements of vitamins and during the remainder they received placebos identical in taste and appearance to the vitamin capsules.

In the first experimental series each man reported at the laboratory twice a week for a half-day experiment. In the succeeding experimental series each man reported once a week for a 24-hour experiment. In all cases the experiments consisted of physiological and biochemical observations in a standard state of rest, a standard period and intensity of muscular exertion, followed by physiological and biochemical observations at successive stages of recovery. During the actual exercise the pulse rate was continuously recorded.

TABLE 1

Duration of exercise in minutes and the average external work accomplished per experiment, in kilogram meters, for the several experimental series.

TIME	SERIES I		SERIES II		SERIES III		SERIES IV	
	Time	Kg. M.	Time	Kg. M.	Time	Kg. M.	Time	Kg. M.
Afternoon	15	14,490	90	55,150	90	57,280	90	57,360
Morning	—	———	120	73,430	90	57,280	90	57,360

The exercise consisted in marching for a definite period on the motor-driven treadmill at a constant speed of 3.85 miles per hour and a 12.5% angle of climb in the first series and 10% in all subsequent series. Standard packs were carried and the clothing, time of day, relation to previous meals and exertion were standardized. The exercise room was maintained at 78° F., with relative humidity between 40 and 60% saturation. The exercise was severe enough to be somewhat beyond the capacity of the average untrained college student. The duration of exercise and external work accomplished are shown in table 1.

In order to eliminate training effects a preliminary period of 2 to 4 weeks in each experimental series was devoted to

training. To ensure that training and other extraneous effects would be at a minimum the men in each experimental series were placed in two groups, one of which received the vitamin supplements during the first half of the experimental period proper while the other group received the vitamins in the second half of the period. The supplements used are shown in table 2.

TABLE 2

The daily vitamin supplements, in mg. The daily administration of the capsules was checked at the Fort Snelling Dispensary.

	THIAMINE Cl (B_1)	NICOTINIC ACID AMIDE	CALCIUM PANTO-THENATE	RIBOFLAVIN (B_2)	PYRIDOXINE (B_6)	ASCORBIC ACID (C)
Series 1	5	100	—	—	—	100
Series 2	7	100	20	10	10	200
Series 3	17	100	20	10	10	200
Series 4	—	—	—	—	100	—

The physiological and biochemical variables, which were measured to assess the results of the exercise and the effects of the vitamins on them, included: the pulse rate, heart size, stroke output of the heart, oxygen consumption, respiratory quotient, urinary nitrogen and ketone body excretion, and concentration in the blood of lactate, sugar hemoglobin and ketone bodies.[2] In experimental series III and IV total urinary excretion of ketone bodies and of nitrogen was measured and 2-hour sugar tolerance curves were obtained at the end of the exercise.

[2] The following methods were used: heart rate — integrating cardiotachometer; heart size and stroke output — roentgenkymographic method of Keys and Friedell ('39), and Keys et al. ('40); oxygen consumption and respiratory quotient — collection and measurement of expired air in a compensated gasometer and direct Haldane analysis of the gas samples; blood hemoglobin — acid hematin method with the Evelyn photo-electric colorimeter; blood sugar — Folin-Wu modification of Benedict's method with the Evelyn colorimeter (Folin-Wu, '20; Folin, '29); blood lactate — Edward's modification of the method of Friedeman, Cotonio and Schaeffer (Edwards, '38); blood acetone and other ketone bodies — method of Barnes and Wick ('39); blood bisulfite binding substances — method of Schrader ('40); urinary nitrogen — direct micro-Kjeldahl method; urinary acetone and other ketone bodies — method of Van Slyke ('17, '29).

RESULTS

The detailed results of the several experimental series are too extensive for publication here. Grand averages for some of the more significant variables are given in tables 3 and 4.

Only one man reported a marked change in his ability to carry out the heavy exercise but his subjective improvement occurred during his period on the placebos. Other subjective reports of slight improvement occurred equally in placebo and vitamin supplement periods.

The objective measurements showed that the vitamin supplementation had no effect either on the stroke output of the heart or on the heart rate before, during or after exercise. The heart size was slightly reduced, on the average, after the heavy work as compared with the size in previous rest, but this small change was shown in both placebo and supplement periods.

The average concentration of lactic acid in the blood in series I was increased almost exactly threefold by the exercise but this was unaffected by the vitamin supercharging. The rate of removal of lactic acid from the blood in recovery was likewise independent of the vitamin supplementation. In all the other experimental series the blood lactate level remained relatively constant before and after the work period both on placebos and on vitamin supplements.

The bisulfite binding substances in the blood were measured before and after work in the first experimental series but no change was found in either placebo or vitamin supplement periods.

The blood sugar was decreased by the work in all the experiments by average amounts of 14.1 to 18.4 mg. per 100 cc. but this decrease was not different in the vitamin supplement periods as compared with the placebo periods. Blood sugar measurements in series II and III showed, on the average, a somewhat more rapid return of blood sugar toward normal values during recovery in the vitamin supplement periods but the difference was not statistically certain. In series I, in

TABLE 5

Grand averages for some significant variables.

Blood Lactic Acid, mg. per 100 cc.

EXPERIMENTAL SERIES I

Period on	Rest	After Work 2 min.	After Work 10 min.	K[1]
Vit.	11.6	33.2	24.2	0.105
Plac.	10.6	33.2	23.0	0.089

EXPERIMENTAL SERIES II

Period on	Rest	End of Work	10 min. Recovery
Vit.	8.53	12.16	11.33
Plac.	8.76	10.85	10.39

Heart Rate, Beats per min.

EXPERIMENTAL SERIES I

Period on	Rest Seated	1st 30 sec. Recovery	9th min. Recovery
Vit.	82.5	147.6	103.2
Plac.	81.5	148.1	106.0

EXPERIMENTAL SERIES II

Period on	Rest Seated	Last min. Work	2nd min. Recovery
Vit.	63.6	150.4	117.7
Plac.	67.1	150.9	114.2

Blood Sugar, mg. per 100 cc.

EXPERIMENTAL SERIES I

Period on	Rest	After Work 2 min.	After Work 10 min.
Vit.	101.0	86.0	84.8
Plac.	96.9	80.8	85.1

EXPERIMENTAL SERIES II

Period on	Rest *	End of Work	10 min. Recovery
Vit.	90.3	72.9	82.5
Plac.	94.5	76.1	86.2

Heart Size and Stroke

Size in sq. cm. frontal projection area in systole, stroke in cc. net systemic output.

Period on	Rest Size	Rest Stroke	3 min. after work Size	3 min. after work Stroke
Vit.	105.7	63.6	104.4	62.9
Plac.	107.3	63.6	104.5	62.6

Blood Hemoglobin, gm. per 100 cc.

Period on	Rest	End of Work	10 min. Recovery
Vit.	13.9	14.9	14.2
Plac.	14.0	15.0	14.4

Dextrose Tolerance, mg. per 100 cc.

Period on	Rest	0.5 hr.	1 hr.	1.5 hr.	2 hr.
Vit.	90.3	161.6	158.8	150.6	140.5
Plac.	94.5	164.1	162.5	161.7	135.7
No work	92.1	128.7	108.8	95.8	78.4

Oxygen Consumption and R. Q.

O_2 consumption in cc. at S. T. P. per min.

Period on	Rest before work O_2	Rest before work R. Q.	10–20 min. after work O_2	10–20 min. after work R. Q.	25–35 min. after work O_2	25–35 min. after work R. Q.
Vit.	263.9	0.845	313.8	0.794	278.2	0.753
Plac.	263.9	0.849	309.0	0.764	291.5	0.770

[1] $K = \frac{2.303}{\Delta t} (\log_{10} C_1 - \log_{10} C_2)$,

where Δt is time in minutes between recovery blood samples, and C_1 and C_2 are concentrations in the 2 recovery blood samples.

TABLE 4

Grand averages for some significant variables.

Blood Sugar, mg. per 100 cc.

	Experimental Series III			Experimental Series IV		
Period on	Rest	End of Work	10 min. Recovery	Rest	End of Work	10 min. Recovery
Vit.	93.6	79.5	86.1	92.6	81.3	85.1
Plac.	94.2	80.1	86.6	85.7	79.3	82.7

Dextrose Tolerance, mg. per 100 cc.

	Experimental Series III					Experimental Series IV				
Period on	Rest	0.5 hr.	1 hr.	1.5 hr.	2 hr.	Rest	0.5 hr.	1 hr.	1.5 hr.	2 hr.
Vit.	93.6	140.1	151.8	163.0	136.1	92.6	132.4	146.8	129.6	107.5
Plac.	94.2	133.8	149.6	155.3	128.8	85.7	130.5	150.4	137.1	129.3

Pulse Rates, Beats per minute

	Experimental Series III			Experimental Series IV		
Period on	Rest Standing	Last min. Work	2nd 60 sec. Recovery	Rest Standing	Last min. Work	2nd 60 sec. Recovery
Vit.	90.6	143.1	102.4	77	136	92.5
Plac.	88.0	146.4	104.2	75	135.5	93.5

Urinary Nitrogen and Ketone Bodies

Total elimination in 14 hours, from 10 P. M. to noon, including morning work. Nitrogen in gm., ketone bodies as acetone in mg.

	Experimental Series III		Experimental Series IV	
Period on	Nitrogen.	Ketone bodies	Nitrogen	Ketone bodies
Vit.	5.84	36.3	7.6	37.4
Plac.	5.67	37.7	5.4	34.3

Oxygen Consumption and R. Q.

O_2 consumption in cc. at S. T. P. per min.

Period on	Rest before work		10–20 min. after work		25–35 min. after work	
	O_2	R. Q.	O_2	R. Q.	O_2	R. Q.
Vit.	243.1	0.811	290.4	0.795	263.1	0.771
Plac.	242.0	0.813	304.3	0.791	253.3	0.761

Blood Acetone, mg. per 100 cc.

Period on	10 min. after work	0.5 hr. after work	1.5 hr after work
Vit.	4.5	4.2	1.9
Plac.	3.7	3.6	2.7

which the severe exercise lasted only 15 minutes, the reverse was observed on the average, but the difference was small.

In all experiments the hemoglobin concentration in the blood rose as a result of the exercise (average 7.1%). This hemoconcentration was independent of vitamin supplementation. The rate of oxygen consumption in rest before work and in recovery was not appreciably affected by the vitamin supplementation.

There was no apparent effect of the vitamin supplement on the respiratory quotient in these experiments although the R.Q. declined to values averaging below 0.80 in series II and III.

Sugar tolerance tests made at the end of work showed a pronounced departure from the normal results obtained on the same men on control days without work. The severe work produced a metabolic state resembling mild diabetes mellitus with some ketosis. However, there was no difference qualitatively or quantitatively in the appearance of these phenomena during either the placebo or vitamin supplemented periods.

The total elimination in the urine of nitrogen and of ketone bodies was measured in series III and IV. The average results were almost identical in the periods on vitamin supplementation and on placebos.

DISCUSSION

In 1934 Baëna injected normal rats with a mixture of ascorbic acid and adrenal cortical extract and reported an increased endurance to muscular strain which was not evident when the cortical extract was used alone. Brack ('36) reported improved muscular performance with ascorbic acid plus cortical extract in isolated muscles but found that the ascorbic acid itself was without effect. Wachholder and Podesta ('37), Guarnaschelli-Raggio ('38), and Sievers ('39) reported somewhat discordant results on the working capacity of isolated frog muscles bathed in ascorbic acid solutions.

The reports on the action of excess thiamine chloride are likewise difficult to interpret. Minz and Agid ('37) and Hano ('37) stated that the working capacity of muscle is improved by injections of thiamine. Briem ('39) claimed that the addition of thiamine to the medium bathing isolated muscles increased the number of contractions induced by acetylcholine. Kaiser ('39) denied this and explained Briem's results on the basis of neglect of control of the acidity of the medium.

In the course of studies in induced thiamine deficiency Williams, Mason, Wilder and Smith ('40) obtained some impressions that an unusually large intake of thiamine may promote a degree of physical vigor not seen on an ordinary intake of the vitamin. Gounelle ('40) gave 10 to 15 mg. of thiamine chloride daily to cyclists and football players with results in competition that were considered favorable.

Morell ('40) reported improved endurance in bicycle tests with two men who received 100 gm. of a vitamin mixture as they were approaching exhaustion. The mixture contained, in each 100 gm., 62 gm. dextrose, 100 mg. nicotinic acid, 11.7 mg. thiamine chloride, and 200 mg. ascorbic acid. These results are readily explained by the additional supply of carbohydrate fuel.

Analysis of the garrison rations supplied at Fort Snelling during the 10 months of the present study was made on the basis of the stock purchase, consumption records and daily menus. The grand average daily intake per man on these garrison rations was 1.7 mg. of thiamine chloride, 2.4 mg. of riboflavin, and 70 mg. of ascorbic acid. The actual food consumption records are on file in this laboratory.

Vague statements in the literature to the effect that vitamin supplementation of "adequate" diets may promote increased well-being and vigor need not be discussed. Sherman and Campbell ('37) offer experimental data on rats. Actually, however, the latter results only show that a laboratory diet which allows reproduction and freedom from nutritional disease does not necessarily promote maximal growth.

SUMMARY

Soldiers maintained on standard U. S. Army garrison rations were repeatedly subjected to standardized severe exercise on the motor-driven treadmill. Circulatory, metabolic and blood chemical responses were measured.

In four series of studies involving 256 experiments, large daily supplements of thiamine chloride, riboflavin, nicotinic acid, pyridoxine, pantothenic acid and ascorbic acid were administered over periods of 4 to 6 weeks alternating with equal periods of placebo administration.

In neither brief extreme exercise nor in prolonged severe exercise and semi-starvation were there indications of any effects, favorable or otherwise, of the vitamin supplementation on muscular ability, endurance, resistance to fatigue or recovery from exertion.

Healthy young men expending an average of 3700 to 4200 calories per day are not benefited by a daily supply of more than 1.7 mg. thiamine chloride, 2.4 mg. riboflavin, and 70 mg. of ascorbic acid.

It is concluded that no useful purpose would be served by enrichment of present U. S. Army garrison rations with the vitamins studied.

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SERUM PHOSPHATASE IN EXPERIMENTAL SCURVY

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TWO FIGURES

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INTRODUCTION

The relationship between phosphatase activity and ascorbic acid has been the subject of considerable investigation. It has been adequately demonstrated that ascorbic acid does not activate alkaline phosphatase in vitro (King and Delory, '38; Thannhauser et al., '37, '38; Albers, '40). However, in vivo experiments with guinea pigs (Scoz et al., '37; Todhunter and Brewer, '40) indicate that the withdrawal of ascorbic acid from the diet results in a lowering of serum alkaline phosphatase activity. In infantile scurvy the serum phosphatase is reduced, and rises after the administration of ascorbic acid (Shwachman, '41).

The present investigation has been undertaken to determine further the relationship in vivo between serum phosphatase activity and scurvy with a view to elucidating the more exact role of ascorbic acid. The activity of serum phosphatase was determined during the development of scurvy, when manifest symptoms of the disease were present, and during treatment with varying amounts of ascorbic acid. Estimations were made of the serum "acid" phosphatase levels of normal and scorbutic animals since no data have been reported on the possible variation in scurvy of other phosphatases than the "alkaline."

METHODS

Guinea pigs [1] 6 to 7 weeks of age and weighing from 250–275 gm. were separated into groups of two or three and housed in clean wire cages. The animals were fed a scorbutogenic diet consisting of equal parts of skim milk (heated at 90°C. for 12 hours), rolled oats and bran fed ad libitum. The diet was supplemented by 1 cc. of cod liver oil every 4 to 5 days. Certain animals were not given any ascorbic acid. To others, a measured amount of ascorbic acid dissolved in water immediately before use was fed by dropper. Animals fed the scorbutogenic diet, but no ascorbic acid, invariably showed clinical evidence of scurvy in 18–24 days. At autopsy, extensive hemorrhages and fragility of the bones were observed. Histological and roentgenographic [2] confirmation of scurvy was obtained. When supplemented with ascorbic acid the scorbutogenic diet was adequate for normal growth of the animals as was evident from the increase in weight and absence of symptomatology.

Serum "alkaline" phosphatase [3] determination. The animals were bled directly from the heart under light ether anesthesia. Approximately 2.5 cc. of blood were withdrawn for each determination. Todhunter and Brewer ('40) have demonstrated that ether anesthesia under these conditions does not affect the serum phosphatase activity.

Serum "alkaline" phosphatase was estimated by a modification of the Bodansky method (Shwachman, '41) using 0.5 cc. of fresh serum incubated with sodium β-glycerophosphate [4] in veronal buffer at pH 8.6. In addition, $Mg.^{++}$ was added to a final concentration of 0.009 M. The reaction was carried out at 37°C. for 60 minutes. Acid-soluble inorganic phosphate

[1] These animals were made available to us by Mr. E. J. Staff of the Rhode Island Department of Health to whom we express our thanks.

[2] We are indebted to Drs. Sidney Farber, Nathan Rudo, and Theodore Ingalls for the histological and roentgenographical examinations.

[3] "Alkaline" phosphatase, as used in this report, refers to the enzyme whose optimal activity is in the pH range 8.5–9.0. "Acid" phosphatase refers to the enzyme with pH optimal activity at 4.9–5.0.

[4] Obtained from the Eastman Kodak Company.

was determined by the method of Fiske and Subbarow ('25) using a photoelectric colorimeter. The activity is expressed in units, each equivalent to 1 mg. of inorganic phosphorus liberated per 100 cc. of serum under the conditions of the experiment.

Serum "acid" phosphatase. Serum "acid" phosphatase was estimated by the method of Gutman and Gutman ('40) using sodium β-glycerophosphate (0.5%) as the substrate in veronal (0.212%) — acetate buffer at pH 5.0. No activation by $Mg.^{++}$ of the acid phosphate was observed and, therefore, was omitted from the substrate. Incubation was at 37°C. for 3 hours. Since we have confirmed the observation that the rate of glycerophosphate hydrolysis is linear over the 3-hour period, the values are given in units corresponding to the mg. inorganic phosphorus liberated in 1 hour per 100 cc. serum.

EXPERIMENTAL

Serum "alkaline" phosphatase in scurvy. Thirty 6-week-old animals were divided into groups of six animals each and fed the scorbutogenic diet. The diet of one group was supplemented by 1 mg. ascorbic acid daily; a second group received 6 mg. ascorbic acid daily; two groups received no added ascorbic acid. One group was fed a diet of prepared soy meal[5] chow supplemented with fresh vegetables. Estimations of serum phosphatase of half the number of each group were made approximately every 5 days so that each animal was bled once in about 10 days. Near the end of the experiment estimations were carried out more frequently. The results are represented in figure 1.

During the first 5 days there is a marked drop in the "alkaline" phosphatase activity of all the animals except those on the vegetable diet. This drop appears to be due to the refusal of the animals to eat the scorbutogenic diet even when supplemented with large doses of ascorbic acid. Subsequently, the serum alkaline phosphatase of the animals

[5] Purina.

receiving ascorbic acid rises, and then declines slowly with increase in age of the animals. The animals receiving no supplement of ascorbic acid show a continued marked decline in serum alkaline phosphatase to a low level of about 2 units. In one instance the level fell to as low as 0.3 units (fig. 2). In contrast, animals fed the scorbutogenic diet showed no variation in serum acid phosphatase from those on an adequate diet.

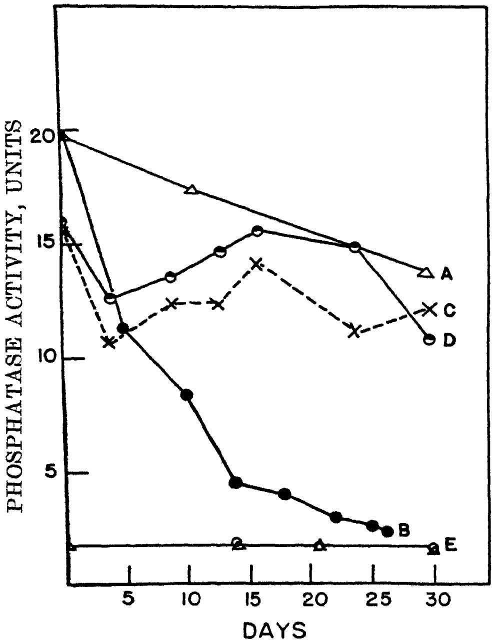

Fig. 1 The serum "alkaline" and "acid" phosphatase activity of normal and scorbutic guinea pigs. Alkaline phosphatase values of guinea pigs fed: A, vegetable diet; B, scorbutogenic diet; C, scorbutogenic diet supplemented with 1 mg. ascorbic acid; D, scorbutogenic diet supplemented with 6 mg. ascorbic acid; E, "acid" phosphatase values of guinea pigs fed vegetable diet, △ and scorbutogenic diet, 0.

Animals fed a fresh vegetable diet supplemented by large doses of ascorbic acid showed no significant differences in phosphatase levels from animals receiving the same diet without an ascorbic acid supplement.

The animals which received the scorbutogenic diet supplemented by 1 mg. of ascorbic acid in most cases maintained almost the same alkaline phosphatase level as the animals receiving 6 mg. daily, although two animals receiving the 1 mg. supplement daily developed mild scurvy (phosphatase values, 3.2 and 4.2 respectively). This indicates that 1 mg. of ascorbic acid daily is a critical dose and that a larger supplement of ascorbic acid is required to maintain the phosphatase level and to protect the animal from scurvy. That 1 mg. of ascorbic acid given daily is a critical dose was also apparent from the change in weight of these animals. The average weight of the animals receiving a supplement of 1 mg. ascorbic acid daily was 285 gm. at the end of 35 days, or an average gain of 17 gm. On the other hand, the final weight of the animals receiving 6 mg. daily was 351 gm. or an average gain of 73 gm. The average loss in weight of the animals on the unsupplemented diet was 59 gm. during the same period. However, in most animals a dose of 1 mg. ascorbic acid daily was as effective as the excessive dose in maintaining the phosphatase at a normal level.

Four 500-gm. guinea pigs were fed the scorbutogenic diet. After 1 month on the diet the serum phosphatase fell from 12.8 units to 2.0 units. This would indicate that the decrease in phosphatase during scurvy is not characteristic of very young animals only.

We have been unable to confirm the observation of Scoz et al. ('37) that there is an initial rise in serum alkaline phosphatase after the withdrawal of vitamin C from the diet, which is then followed by a decline in serum phosphatase. The present data indicate no increases in phosphatase activity, either permanent or transitory at any stage during the development of scurvy.

Effect of "scorbutogenic diet"[6] *on serum alkaline phosphatase of animals (rabbits) which do not develop scurvy.* Bodansky ('34) has demonstrated that modifications of diet

[6] Scorbutogenic diet refers to the diet which, when fed to guinea pigs, produces scurvy.

may induce changes in the serum alkaline phosphatase activity. Although it has been shown that the serum alkaline phosphatase activity of scorbutic animals is restored to normal levels solely by the administration of ascorbic acid, it was deemed desirable to determine whether the scorbutogenic diet itself induced any changes in the serum alkaline phosphatase activity of an animal that is able to synthesize ascorbic acid and does not develop scurvy. Two litters of young rabbits, one litter 31 days old and the other 50 days old, were divided and half the number in each litter placed on

TABLE 1

The effect on the serum phosphatase activity of feeding an ascorbic acid deficient diet to young rabbits.

RABBITS	INITIAL AGE	INITIAL SERUM PHOSPHATASE	ON DIET	FINAL SERUM PHOSPHATASE
	weeks	*units*	*weeks*	*units*
N1-a [1]	4½	17.1	4½	9.6
N1-b	4½	14.7	4½	10.7
S1-a	4½	17.8	4½	11.4
S1-b	4½	15.7	4½	13.2
N2-a	7	7.0	3½	6.3
N2-b	7	9.6	3½	6.9
S2-a	7	9.2	3½	8.5
S2-b	7	9.7	3½	6.0

[1] N: refers to an animal on a diet consisting of purina rabbit chow supplement with fresh green vegetables.

S: refers to animals fed the ascorbic acid deficient diet described previously. Numeral indicates litter.

the "scorbutogenic" diet while the other half was fed a diet of chow and fresh vegetables. The animals on the "scorbutogenic" diet gained very little weight as compared with those on the commercial food. The serum phosphatase values determined after 3½ and 4½ weeks (table 1) indicate that the "scorbutogenic" diet in itself has no apparent effect on serum alkaline phosphatase.

Evidence that low serum phosphatase activity in scurvy is due to absence of the enzyme. The question arises as to whether the low phosphatase activity in severe scurvy is due

to the absence of some activator essential for phosphatase activity, to the presence of some inhibitor, or to a quantitative alteration in the enzyme.

If the lowering of serum phosphatase of scorbutic animals and the rise after treatment with ascorbic acid were due to an alteration in activator or inhibitor concentration, the addition of a high activity serum to a low activity serum would result in a disproportionate change in resultant activity. If the low activity is due to the presence of an inhibitor the mixture of low and high activity sera should lead to a disproportionate lowering of activity. If activity is dependent on the concentration of enzyme the resultant activity should equal the sum of the separate activities. Experiments were carried out using mixtures of extremely low activity sera (scorbutic) and high activity sera (young normal) made up of varying proportions of each. No activation of the phosphatase in low activity sera by high activity sera or inhibition of high activity sera by those of low activity could be demonstrated. The observed activity is essentially equal to the sum of the activity calculated from the activity of each. It appears, therefore, that the change in scurvy involves an actual decrease in the enzyme.

Effect of administration of ascorbic acid on serum alkaline phosphatase of scorbutic guinea pigs. Fifty-five animals were fed the scorbutogenic diet for 18–20 days, after which time practically all showed clinical signs of scurvy. Serum phosphatase estimations were made and the animals were then fed the scorbutogenic diet supplemented with varying amounts of ascorbic acid daily. Estimations of the serum alkaline phosphatase were carried out periodically. The results shown in figure 2 represent the response for individual animals. Most striking is the prompt increase in serum phosphatase after the administration of ascorbic acid and the decrease which follows the withdrawal of ascorbic acid. This is best illustrated by animals 3, 4, 10A and 15A (fig. 2). The speed of the response is illustrated by animals S10 and S12 where, 24 hours after administration of ascorbic acid, a small increase

is apparent, and after 48 or 72 hours the increase is more marked. An examination of the data of animal 10A (fig. 2) which was given a minimal amount of ascorbic acid indicates that the rise is slow but eventually as great as that obtained with large doses in other scorbutic animals. If the supple-

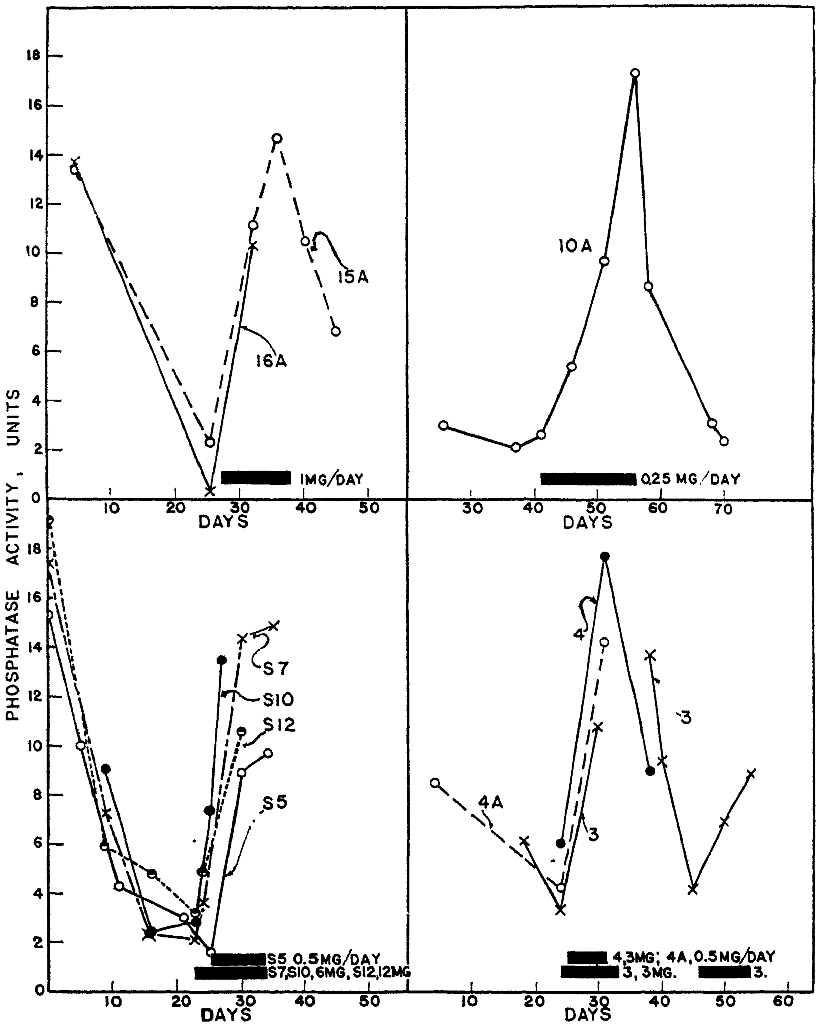

Fig. 2 The effect of ascorbic acid on the serum alkaline phosphatase in scorbutic guinea pigs.

ment of ascorbic acid is increased to 0.5 mg. daily (S5 and 4A) the rise becomes immediate. The fall in phosphatase activity is equally prompt when ascorbic acid is withdrawn from the diet. An examination of figure 2 shows clearly that the increase in phosphatase activity is practically independent of the dosage. These observations would suggest that the increase in phosphatase activity is not due to a direct activation of the enzyme by ascorbic acid in the circulating blood but rather to the stimulation of phosphatase-producing centers (Gould and Shwachman, '42). This rapid change is probably a reflection of the histological response which has been shown by Wolbach and Howe ('26) to be definite in 24–48 hours. More direct evidence to support this view is our failure to obtain activation of the low serum phosphatase from scorbutic animals with ascorbic acid in vitro.

It is noteworthy that large doses of ascorbic acid administered to normal animals have no appreciable effect in raising the phosphatase level, whereas small doses administered to scorbutic animals will result in a marked increase in the serum phosphatase activity. Often the resulting response may be higher than the normal level. This has likewise been observed in infantile scurvy (Shwachman, '41).

In the course of the present study no scorbutic animal failed to show either an increase in serum phosphatase after the administration of ascorbic acid or a decline after withdrawal of the vitamin.

"Acid" phosphatase. Twenty guinea pigs, consisting of sixteen 6 weeks old and four adult 500-gm. animals, were divided into groups some of which were fed a diet of chow and fresh green vegetables, and others were given the scorbutogenic diet. At intervals the "alkaline" and "acid" serum phosphatase activities on the samples were determined. The results (fig. 1) indicate quite clearly that while there is a pronounced decline in the "alkaline" phosphatase activity of the sera of scorbutic animals, there is no change in the activity of the "acid" phosphatase. Moreover, while there is a regular decline in the serum "alkaline" phosphatase ac-

tivity of normal animals with increase in age, there appears to be no corresponding decline in the "acid" phosphatase activity.

Blood acid soluble inorganic phosphate. The method employed for the estimation of phosphatase activity involves in every analysis a control for the inorganic phosphate of the serum. The values obtained for normal and scorbutic animals have been compared and no significant difference could be established.

SUMMARY AND CONCLUSIONS

1. Guinea pigs fed a scorbutogenic diet show an immediate and continued decline in "alkaline" serum phosphatase activity reaching extremely low levels.
2. Animals (rabbits) able to synthesize ascorbic acid show no decline in serum alkaline phosphatase when fed a scorbutogenic diet.
3. Scorbutic guinea pigs fed a scorbutogenic diet supplemented by from 0.25–12.0 mg. ascorbic acid daily show rapid increases in serum alkaline phosphatase activity and rapid decreases when ascorbic acid is withdrawn.
4. Ascorbic acid does not activate the low serum phosphatase of scorbutic guinea pigs in vitro. The effect in vivo is due to an increase in enzyme production during recovery from scurvy.
5. There is no apparent change in serum "acid" phosphatase or in the serum inorganic phosphate of scorbutic guinea pigs.

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QUANTITATIVE DISTRIBUTION OF PHOSPHORUS AND CALCIUM IN CERTAIN FRUITS AND VEGETABLES

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The quantitative distribution of phosphorus and of calcium in the fruits and vegetables which we use as food is of scientific significance both with regard to our own nutrition and to our understanding of the nutritional processes of plants. In turn, any knowledge which we may gain of the quantitative behavior of these elements in plant nutrition should help in the planning and interpretation of studies of the extent to which the calcium and phosphorus contents of foods may be influenced by cultural conditions.

The idea — sometimes called Liebig's "law of the minimum" and apparently confirmed by Claude Bernard's dictum of the fixité of the internal environment — that the chemical composition is essentially constant for a given organ of a given species, is now seen to have been an over-simplification. Within a given species chemical composition may be measurably influenced both by environmental and genetic factors. Because of limitations of space we here cite but two of the recent investigators of such variations.

Miller ('38) while citing many observations to show that the older views were too rigid still states it as common knowledge that plants of different species grown under identical conditions differ in their elementary composition; and Hoagland and coworkers also show species differences in the relative amounts of chemical elements which plants absorb,

although the amount absorbed and its effect on growth and reproduction are also largely influenced by the relative concentrations, and the interactions, of the ions present (Hoagland, '40; Hoagland and Arnon, '41).

That natural and induced differences in chemical composition of tissues may be more significant than science had until recently supposed is a fact of both theoretical and practical importance. Also it is important that this fact be not exaggerated, as has happened when extreme instances were cited as if they were representative.

The investigation reported in the present paper deals (1) with presumably representative specimens of foods from various sources as they actually reach the consumer, i.e., with all inedible parts removed, and (2) with the quantitative distribution of the calcium and phosphorus of such foods among the structural parts of food plants.

Concentration of phosphorus in seeds

It is well known that plants tend to concentrate phosphorus in their seeds; calcium, in their leaves. Thus in presumably typical cases of the corn plant it has been found that at maturity 75% of the total phosphorus was in the seeds, while 58% of the total calcium was in the leaves (Latshaw and Miller, '24; Miller, '38).

Our studies here reported include (1) determinations of phosphorus in the seeds compared with the flesh (pulp and juice) of blackberries, raspberries, currants, grapes, tomatoes, okra, and squash; (2) studies of the calcium and phosphorus contents of the seeds versus the pods of four varieties of beans representing different stages of maturity; and (3) similar comparisons of the calcium and phosphorus contents of the flowerbuds (the first step toward formation of seeds) of broccoli in comparison with the adjacent leaves and edible portions of the stems of the same plants.

The data of the first of the three series of studies just mentioned are summarized in table 1.

All of the work summarized in table 1 — the gathering or purchase of the food, the preparation of the sample, and every step of the analytical determination — was done by the same person (M.S.R.) so that the "personal equation" was the same throughout. The specimens analyzed were presumably representative samples of the respective kinds of food as

TABLE 1

Phosphorus contents of entire edible portions of fruits, of the same with seeds removed, and of the seeds alone

DESIGNATION	PHOSPHORUS, PER CENT OF FRESH WEIGHT			PERCENTAGE OF TOTAL P IN SEEDS	WEIGHT OF SEEDS AS PER CENT OF WHOLE FRUIT
	Whole fruit	Without seeds	Seeds		
Blackberries I	0.034	0.018	0.066	64	33
" II	0.030	0.018	0.085	71	29
" III	0.027	0.013	0.086	75	30
" IV	0.028	0.015	0.082	61	21
" V	0.032	0.018	0.097	44	15
" VI	0.025	0.011	0.064	54	21
" VII	0.029	0.013	0.113	50	13
" VIII	0.031	0.015	0.083	72	27
" Average	0.030	0.013	0.085		
Raspberries I	0.036	0.027	0.070	25	13
" II	0.037	0.028	0.076	33	16
" III	0.035	0.028	0.076	30	14
" IV	0.032	0.025	0.066	31	15
" Average	0.035	0.027	0.072		
Currants	0.036	0.018	0.132	63	17
Grapes (Tokay)	0.021	0.020	0.065	7	2.4
Tomatoes I	0.029	0.024	0.203	14	2.1
" II	0.027	0.025	0.070	12	4.5
" III	0.020	0.017	0.074	16	4.4
" IV	0.027	0.024	0.094	14	4.1
" V	0.023	0.019	0.107	21	4.6
" VI	0.025	0.021	0.138	18	3.3
" VII	0.024	0.018	0.136	13	2.4
" Average	0.025	0.021	0.117		
Okra	0.060	0.053	0.112	25	14.0
Squash	0.021	0.014	0.038	56	32.0

actually reaching consumers under present-day marketing conditions. The foods in this series are all of kinds which may be eaten either with or without their seeds, so that the quantitative distribution of the phosphorus as between the flesh and seed is of scientific significance as bearing upon the amounts of phosphorus presumably available in human nutrition, and also the plant-nutrition problem of the extent of the concentration of the phosphorus of the fruit into its seed. In the cases of blackberries, raspberries, and tomatoes, where from four to eight entirely separate specimens of each of these three species of fruit were analyzed, the extent of the variations here found is presumably representative of the variability of these foods as they are actually encountered in the general food supply, and should give correspondingly more valid impressions of the reliability of the foods as sources of phosphorus in the nutrition of the human population than would the data of an investigation whose object was to find the greatest variation which could be discovered or experimentally induced.

Hence, even for the numbers of cases here reported, it is of interest to note the average deviations, and whether these are greater for the fruits with or without their seeds.

There follow brief statements regarding our findings upon different foods listed in the same order as in table 1.

Blackberries contained about two-thirds of their total phosphorus in their seeds, where the percentage of phosphorus was about five times as high as in the flesh (pulp and juice) of the same fruits. The average deviation was 0.0022 (7% of the mean) in the blackberries with, and 0.0029 (22% of the mean) in the berries without, their seeds. The phosphorus content without the seeds is therefore somewhat the more variable; but it doubtless represents more nearly the amount actually available in human nutrition.

In raspberries the per cent of phosphorus in the seeds was almost three times that in the pulp and juice, but less than one-third of the total amount of phosphorus in this fruit was in its seeds. The average deviation was about the same pro-

portion (only about 4%) of the mean value for the fruit with or without the seeds. Probably only a negligible part of the contents of the seed is nutritionally available to man.

In currants 63%, and in grapes only 7% of the total phosphorus was here found in the seeds.

Tomatoes, with their larger proportion of pulp and juice, contain therein well over four-fifths of their total phosphorus, and less than one-fifth in the seeds even though the concentration is six times as high in the seeds as in the flesh of the fruit. The average deviation was 9% of the mean phosphorus content of tomato with, and 11% of that of tomato without, the seeds. Tomatoes as they reach consumers, even in a large city drawing its supply from widely different sources, are thus of very satisfactorily constant phosphorus content.

In the single samples here analyzed, okra contained 25%, and summer squash 56%, of its total phosphorus in its seeds.

It is a safe generalization that foods should be analyzed without their indigestible seeds if the analysis is to show what the food actually furnishes to the nutritional process, and that the exclusion of the seeds widens the calcium/phosphorus ratio.

On the other hand, there are foods in whose development the seed becomes the part of most significance for our nutrition. Thus we eat snap beans when the seed is so immature that more than nine-tenths of the edible portion is pod and the calcium/phosphorus ratio of this entire edible portion is fairly wide (1.46 in the first item of table 2); whereas this ratio is narrow in the phosphorus-rich mature seed (0.18 in the last item of the same table).

Calcium and phosphorus in pods and seeds of beans

Calcium and phosphorus were determined in pods and seeds (ovules) of several varieties of beans, some of them at different stages of maturity. These data are summarized in table 2. As the differences are due in part to variety and in part to maturity, full discussion would require too much space. It suffices to note that the order of the specimens in the table is that of increasing maturity and that the last column clearly

TABLE 2

Calcium and phosphorus in pods and ovules of beans

DESCRIPTION	SEEDS, PER CENT OF TOTAL WEIGHT	WHOLE		PODS		SEEDS		PERCENTAGE OF THE TOTAL P IN THE SEEDS
		Ca %	P %	Ca %	P %	Ca %	P %	
Wax (early snap stage)	5½	0.057	0.039	0.063	0.030	0.053	0.147	22
Green stringless	7	0.044	0.037	0.045	0.024	0.046	0.139	32
" "	9½	0.043	0.039	0.046	0.032	—[1]	0.134	30
Cranberry, green	41	0.095	0.110	0.120	0.013	0.051	0.214	91
" yellow	44	0.112	0.113	0.167	0.012	0.055	0.203	93
" pink-striped	52	0.125	0.102	0.215	0.008	0.046	0.200	96
Black-eye	52	0.059	0.087	0.048	0.018	0.057	0.133	89
"	80	0.055	0.181	0.168	0.022	0.033	0.188	97

[1] Not determined.

shows a trend to increased concentration of phosphorus in the seed as it matures. The ratio of calcium to phosphorus is higher in the pods than in the seeds, with rather wide variations in a small proportion of the samples.

The apparent increase in calcium in the pods with maturity would indicate that the calcium migrated from plants to pods but not from pods to seeds. This association of high calcium content with tougher pods would seem to indicate increase of pectin and hemicellulose with maturity as found by other investigators (True, '22; Buston, '35).

Broccoli illustrates another type of case, three organs of differing calcium/phosphorus ratios being offered together in the edible portion; e.g., in the two specimens which were here analyzed for both calcium and phosphorus, the ratios were: Leaves , 3.4 and 3.7; edible stems, 1.7 and 3.2; flowerbuds, 0.5 and 0.4.

Distribution between leaves, flowerbuds, and edible stems of broccoli

Four samples of broccoli were purchased at different times in New York City. Including in the samples for analysis as much of the stem in each case as could reasonably be con-

sidered edible, and all the leaves belonging to this portion of the stem, the stems constituted nearly half, the leaves about one-fourth, and the flowers about one-fourth of the total material. In all cases the different parts were analyzed separately for phosphorus, and in two cases for calcium also. The results are summarized in table 3.

TABLE 3

Phosphorus (and calcium) in three edible parts of broccoli

SAMPLE NO.	ELEMENT	ENTIRE EDIBLE PORTION	LEAVES	EDIBLE STEMS	FLOWER-BUDS	FLOWERBUDS AS PER CENT OF TOTAL E. P.	
						Weight	Phosphorus
I	Phosphorus %	0.060	0.064	0.035	0.107	25	45
II	Phosphorus %	0.063	0.070	0.040	0.104	24	40
	Calcium %	0.110	0.237	0.068	0.052		
III	Phosphorus %	0.056	0.055	0.033	0.102	23	43
	Calcium %	0.118	0.202	0.105	0.043		
IV	Phosphorus %	0.060	0.071	0.031	0.095	34	53

The mean phosphorus contents with their average deviations (and the latter as percentage of the former) are respectively: Whole edible portion, 0.060 ± 0.0018 (av.d. = 3.0%); leaves, 0.064 ± 0.0055 (av.d. = 8.5%); edible stems, 0.035 ± 0.0028 (av.d. = 8.0%); flowerbuds, 0.107 ± 0.0035 (av.d. = 3.4%). There is a well-marked concentration of phosphorus in the flowerbuds, and a still more pronounced concentration of calcium in the leaves.

Distribution of calcium in the edible portion of the orange [1]

Formerly, in compiling analyses from the literature, data for the edible portion and for the juice of oranges were tabulated together; but as data accumulated and especially when we were privileged to see unpublished findings of Thomas and Bailey on orange juice obtained by pressure alone, we were led to a study of the quantitative distribution of the calcium be-

[1] This part of the investigation was aided by a research grant to Columbia University by the Florida Citrus Commission.

tween the septa and the more fluid part (juice and soft pulp) of the edible portion. Our experimental findings on this point are summarized in table 4. The ten lots included oranges of large, medium, and small sizes and grown in different regions.

TABLE 4

Calcium in oranges: whole edible portion, juice, and septa

LOT NO.	CALCIUM CONTENT OF:			PERCENTAGE OF TOTAL CA IN SEPTA
	Whole E. P.	Juice with soft pulp	Septa	
	per cent	*per cent*	*per cent*	
I	0.044	0.011	0.164	70
II	0.046	0.012	0.183	75
III	0.044	0.011	0.180	77
→IV	0.040	0.012	0.159	75
V	0.046	0.011	0.118	72
VI	0.046	0.012	0.159	75
VII	0.045	0.011	0.156	75
VIII	0.034	0.015	0.081	70
IX	0.045	0.011	0.167	70
X	0.043	0.010	0.167	70
Average	0.043 ± 0.0027 [1]	0.012 ± 0.0010 [1]	0.153 ± 0.0218 [1]	73 ± 3 [1]

[1] Average deviation.

The average deviations of the calcium contents of the respective parts when stated as percentage of the mean in each case are only: in the whole edible portion, 6.3%; in the juice and pulp, 8.3%; in the septa, 14.2%. With such degrees of constancy in each part, the finding that the septa contain 73 ± 3% of the calcium of the whole edible portion of the orange may be regarded as definite. When one eats the entire edible portion of an orange (septa included) the calcium/phosphorus ratio, as illustrated by our determinations here reported for calcium and compiled data for phosphorus, is 2.0; whereas if the orange is taken as "juice" the ratio is about 0.8.

SUMMARY

Quantitative studies have been made of the relative amounts of phosphorus in the flesh and seeds of seven species of fruits;

in the pods and ovules of four varieties of beans; in the leaves, the edible stems, and the flowerbuds of broccoli; and of calcium in the same parts of broccoli, and in the flesh (juice and pulp) and septa of oranges.

In the course of this work, either phosphorus or calcium was determined in from four to ten entirely separate specimens each, obtained through ordinary marketing channels in a city supplied from widely varied sources, of twelve foods representing edible stems, leaves, flowerbuds, fruits, and edible seeds. In only one-sixth of these foods was the average deviation in phosphorus or calcium content as much as one-tenth of the mean.

Allowing for the extent of the variations encountered in fruits and vegetables as actually produced and marketed, the following findings may be regarded as sufficiently established:

In such "small fruits" as blackberries, currants, and raspberries, the phosphorus content is significantly different when the "edible portion" of the fruit is analyzed with and without the seeds.

In the grapes here studied, the seeds show three times as high a percentage of phosphorus as the flesh, but constitute so small a proportion of the whole fruit as scarcely to influence its average composition.

In tomatoes, okra, and squash (fruits commercially classified as vegetables) the phosphorus content of the edible portion was measurably influenced by the inclusion or exclusion of the seeds. To what extent human beings can assimilate the phosphorus of these seeds remains to be studied.

The quantitative aspect of the transfer of phosphorus from pods to ovules in maturing beans is found to be striking.

Also well marked is the concentration of calcium in the septa of oranges as compared with the juice. The latter, even when carrying the softer portions of the pulp, was found to contain only 27% of the calcium of the edible portion.

The data here reported will also add to the material available to investigators of the natural variability of foods.

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EFFECT ON GROWTH AND CALCIUM ASSIMILATION OF CITRIC ACID — POTASSIUM CITRATE MIXTURES [1]

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In an earlier paper (Lanford, '39), experiments were reported in which the addition of a moderate amount of orange juice to a wheat-and-milk diet caused the assimilation by rats of a larger proportion of the calcium in the food mixture. The present series was planned to test whether the mixture of citric acid and citrates in the orange juice was responsible for its effect in increasing the percentage retention of dietary calcium. It seems clear that this was not the case, under the conditions of our experiment. However, inasmuch as other investigators have since reported beneficial effects of citric acid-citrate mixtures on rickets, both experimental (Shohl, '37; Hamilton and Dewar, '37) and clinical (Shohl and Butler, '39; Siwe, '38), and in some (Day, '40; Hathaway and Meyer, '39), but not all (Day, '40), circumstances, on bone ash, it seems desirable to here report our findings.

EXPERIMENTAL

The basal diet was the same as in the experiment with orange juice. It consisted of 5 parts of ground whole wheat and 1 part of dried whole milk, with added sodium chloride equivalent to 2% of the wheat; a mixture which was found to contain 0.202% of calcium, and may be inferred from earlier studies to supply about 0.44% of phosphorus and to have an energy value of 3.73 kilocalories per gram. (The basal diet itself contained citrate equivalent to about 0.02% as citric acid.)

[1] This research was aided by a grant from the Florida Citrus Commission.

The citric acid-potassium citrate supplement was given as 5 cc. daily[2] of a solution[3] containing 1.6% total (free plus combined) citric acid, of which, in accordance with the findings of Hartmann and Hillig ('34) for orange juice, about three-fourths was free (titratable) acid. The amount of citric acid and citrates thus fed to the experimental animals was twice that provided by the orange juice of the earlier series.

Late each afternoon, all animals were given slightly less of the basal diet than they would normally consume overnight, mixed, in the case of the controls, with 5 cc. of distilled water, and, in the case of the experimental animals, with 5 cc. of the acid-citrate solution.[4] This portion was almost invariably found to have been completely eaten by the next morning, when the dry mixture was again offered ad libitum. In the few exceptional instances, the portion remaining had to be scraped loose from the walls of the container, after which it was promptly consumed by the animal. By far the greater part of the basal diet was taken in the moistened form.

The pairs of animals on which this comparison is based consisted of littermates of the same sex, matched as to initial body weight and build. At the start of the experiment the difference in body weight between the two members of each pair averaged 0.5 gm. for the nine pairs of males and 1.0 gm. for the ten pairs of females. All animals were selected from families which had been maintained through several generations on the basal diet. As in the earlier series, the rats were 28 days of age when the experiment was begun, and at 60 days of age were killed and analyzed as described elsewhere (Lanford, '39).

[2] Except on Sundays and on the day preceding that on which the animals were to be killed.

[3] Prepared by dissolving 1.31 gm. of crystalline citric acid ($C_6H_8O_7 \cdot H_2O$) and 0.48 gm. of crystalline potassium citrate ($K_3C_6H_5O_7 \cdot H_2O$) in 100 cc. of solution.

[4] In this series, no attempt was made to dilute the basal diet of the controls with a neutral substance supplying the same number of calories as did the citrate solution, since the latter amounted to less than 0.7% of the total caloric intake, falling therefore well within the experimental error in measuring food consumption.

RESULTS AND DISCUSSION

Interpretation of data. Besides the directly measurable values for food intake, initial body weight, gain in weight during the experimental period, and total body calcium at 60 days of age, given for each animal in table 1, computations based on these data were made for (a) gain per 1000 kilocalories consumed, (b) total calcium retained during the experimental period, and (c) calcium retained as percentage of the intake. In computing (a), an energy value of 471 kilocalories per mol was taken for citric acid (from the International Critical Tables). To determine calcium retention, the calcium content of each animal at the start of the experimental period was estimated from analyses of other 28-day-old animals of the same hereditary and nutritional background, the male and female animals showing respectively an average of 0.714% and 0.738% of calcium in net weight, the net weight averaging 5.0 gm. less than the live weight for both sexes at this age.

The significance of the data has been assessed statistically in two ways: comparison being made both between the corresponding sex and dietary groups, each considered as a unit (with the results in table 1), and between the individual members of each pair, as in table 2. Only by the latter procedure is the advantage of careful initial matching of the pairs fully appreciable in more clear-cut results. Thus, for example, when the data on weight gain from 28 to 60 days of age are treated by the former procedure the difference in performance between the animals which did and those which did not receive the citrate mixture appears nonsignificant; whereas, by the latter treatment an unmistakable difference is shown.

Effect of citric acid — potassium citrate mixtures on calcium retention. Under the conditions of this experiment, administration of a mixture of citric acid and potassium citrate in the molar proportions of 3:1 (thus simulating the citrate buffer mixture of orange juice) was without measurable effect on the percentage retention of the calcium of a wheat-and-milk diet.

TABLE 1

Record of individual rats, with and without acid-citrate supplement.

RAT NO. AND DIET GROUP		FOOD INTAKE		BODY WEIGHT			BODY CALCIUM		
		Basal diet	Citric acid-citrate solution	At 28 days	Gain, 28–60 days		At 60 days	Increase, 28–60 days	
		gm.	*cc.*	*gm.*	*gm.*	*gm./1000 kg.cal.*	*gm.*	*gm.*	*% of intake*
Males									
80689	acid-citrate	258.5	130	40.7	81.3	83.87	0.7449	0.4900	93.83
80690	control	230.0		40.2	64.8	75.53	0.6895	0.4382	94.32
80692	acid-citrate	242.0	130	37.4	75.6	83.28	0.6989	0.4761	97.40
80691	control	253.5		39.0	81.0	85.66	0.7464	0.5036	98.34
80813	acid-citrate	246.0	135	38.6	71.4	77.37	0.7029	0.4632	93.22
80812	control	243.5		38.6	71.4	78.61	0.6935	0.4538	92.25
80829	acid-citrate	284.0	130	43.6	83.4	78.36	0.7855	0.5101	88.92
80830	control	224.5		43.1	60.9	72.73	0.6828	0.4110	90.63
80930	acid-citrate	286.0	135	42.0	87.0	81.15	0.7722	0.5080	87.93
80931	control	285.5		41.5	92.5	86.86	0.7650	0.5044	87.46
80950	acid-citrate	291.0	130	39.4	86.6	79.41	0.7589	0.5133	87.33
80952	control	274.5		38.7	78.3	76.47	0.7130	0.4734	85.37
80960	acid-citrate	273.0	130	40.3	77.7	75.92	0.7248	0.4728	85.73
80961	control	228.5		39.9	60.1	70.52	0.6465	0.3973	86.07
80963	acid-citrate	251.0	130	35.1	75.9	80.63	0.6721	0.4572	90.18
80962	control	219.0		35.5	63.5	77.73	0.6048	0.3870	87.48
80979	acid-citrate	242.5	130	39.5	70.5	77.51	0.7105	0.4642	94.77
80978	control	200.0		39.5	52.5	70.38	0.6235	0.3772	93.37
Mean	*acid-citrate*	*263.8*		*39.6*	*78.8 ±1.4*	*79.72 ±0.60*	*0.7301*	*0.4839 ±0.0049*	*91.03 ±0.87*
	control	*239.9*		*39.6*	*69.4 ±2.8*	*77.17 ±1.32*	*0.6850*	*0.4384 ±0.0111*	*90.59 ±1.00*
Females									
80693	acid-citrate	238.5	130	39.1	62.9	70.30	0.7018	0.4502	93.44
80695	control	227.5		38.4	62.6	73.77	0.6743	0.4276	93.04
80696	acid-citrate	235.0	130	36.1	62.9	71.34	0.6822	0.4525	95.32
80694	control	236.5		37.9	60.1	68.13	0.6917	0.4487	93.93
80699	acid-citrate	253.0	130	43.5	65.5	69.04	0.7468	0.4625	90.49
80700	control	219.5		42.0	51.0	62.29	0.6740	0.4008	90.39
80703	acid-citrate	199.5	130	36.8	48.2	64.34	0.6112	0.3765	93.42
80702	control	206.5		39.5	45.5	59.08	0.6554	0.4008	96.09
80815	acid-citrate	208.0	135	41.0	49.0	62.73	0.6473	0.3814	90.77
80816	control	227.5		40.0	56.0	65.99	0.6812	0.4227	91.97
80818	acid-citrate	234.5	135	38.9	64.1	72.84	0.6893	0.4389	92.66
80817	control	229.0		39.0	60.0	70.24	0.6828	0.4317	93.32
80935	acid-citrate	244.5	135	39.6	60.4	65.85	0.6882	0.4329	87.65
80934	control	212.0		39.5	48.5	61.33	0.6218	0.3672	85.75
80954	acid-citrate	244.0	130	39.0	65.0	71.02	0.6778	0.4269	86.61
80953	control	249.5		38.0	66.0	70.92	0.6746	0.4311	85.54
80955	acid-citrate	251.5	130	37.2	69.8	74.01	0.6936	0.4560	89.76
80957	control	233.5		37.7	60.3	69.23	0.6505	0.4092	86.75
80956	acid-citrate	251.0	130	36.9	67.1	71.28	0.6656	0.4302	84.85
80958	control	235.0		36.0	64.0	72.10	0.6512	0.4224	87.85
Mean	*acid-citrate*	*236.0*		*38.8*	*61.5 ±1.5*	*69.28 ±0.82*	*0.6804*	*0.4308 ±0.0063*	*90.50 ±0.71*
	control	*227.6*		*38.8*	*57.4 ±1.5*	*67.31 ±1.05*	*0.6658*	*0.4162 ±0.0048*	*90.46 ±0.80*

Since the citrates were fed in this experiment at twice the level provided by the orange juice supplement of the earlier study, which unmistakably increased the percentage utilization of calcium on the same basal diet, it seems clear that the beneficial effect of the orange juice cannot be explained in terms of the citrates which it contains. It seems highly improbable that too great amounts of citric acid and citrates were provided by the supplement in this experiment (twice as large as in the orange juice study), and that an increase in the percentage retention of calcium might have been observed at lower levels of intake. The investigators already mentioned who found a beneficial effect of citrates on bone development and other aspects of calcium metabolism under

TABLE 2

Differences between animals receiving citric acid — citrate mixture and own littermate controls.

	DIFFERENCE, CITRATE ANIMAL MINUS CONTROL	
	Mean	Probable error of mean
Gain in body weight, 28–60 days, gm.	+6.6	±1.34
Gain per 1000 kilocalories, gm.	+2.30	±0.635
Calcium retained, total, gm.	+0.0292	±0.0065
Calcium retained, per cent of intake	+0.23	±0.21

more adverse conditions gave many times more citrate than were here used. It is true that, under less favorable conditions for calcium assimilation, the percentage retained might have been improved by the citrate supplement. However, for our observation that, with the same basal diet supplying the calcium, orange juice increased the fraction of that element which was retained whereas the citric acid-citrate supplement did not, the only interpretation seems to be that citrus juice possesses some additional property which favors calcium assimilation.

This effect of the orange juice appears not to have been due to its preponderance of base-forming elements (equivalent to 0.23 cc. N base in the amount given daily), since the citrate

supplement of the present study had a greater "potential alkalinity" (equivalent of 0.31 cc. N base per day), yet was without effect on the proportion of dietary calcium retained.

The total retention of calcium during the experimental period was significantly higher in the animals receiving citrate, who also ate more of the basal diet and made greater gains in weight than did the control group.

Effect of acid-citrate on growth. The gain in weight of the males receiving the citrate supplement averaged 14%, and that of the females, 7% above those of the control groups (all of the animals having been allowed food essentially ad libitum, as explained above). When matched littermates are compared, the difference in growth in favor of the individuals given citrate appears significant, averaging 6.6 gm. with a probable error of 1.34 gm.

Correspondingly, the gain per 1000 kilocalories averaged 2.30 ± 0.635 gm. higher in the animals which received the supplementary citrate. Since, however, a strongly positive correlation between actual gain in weight and gain per 1000 kilocalories was observed in both this (see table 3) and the earlier study (Lanford, '39) a greater gain per 1000 kilocalories in the citrate group should have been expected ipso facto from the greater total gain.

Interrelationships. In table 3 are given values for Pearson's coefficient, *r*, of correlation between several pairs of variables

TABLE 3

Coefficients of correlation, r, for various possible interrelationships, with probable error of r.

GROUP	MALES, CONTROL	MALES, CITRATE	FEMALES, CONTROL	FEMALES, CITRATE
	$r \pm p.e.$	$r \pm p.e.$	$r \pm p.e.$	$r \pm p.e.$
Ca retained, per cent of intake vs. gain, 28–60 days	—0.06 ±0.222	—0.21 ±0.212	—0.26 ±0.196	—0.31 ±0.190
Ca retained, per cent of intake vs. body weight at 28 days	+0.07 ±0.221	—0.32 ±0.199	—0.01 ±0.210	—0.09 ±0.208
Gain per 1000 kilocalories vs. gain, 28–60 days	+0.80 ±0.080	+0.26 ±0.207	+0.89 ±0.043	+0.82 ±0.069
Body Ca, per cent of net weight vs. gain, 28–60 days	—0.80 ±0.080	—0.75 ±0.097	—0.81 ±0.072	—0.72 ±0.101

whose possible interrelationships are of interest to this discussion. Admittedly, nine or ten comparable cases comprise a group too small for a fully satisfactory test of correlation, especially if the degree of correlation is low. Consequently, all that may be concluded from these figures regarding percentage retention of calcium as possibly affected by body weight at 28 days or by rate of gain in weight is that, if there be any interrelationship, it is probably of low order and doubtful significance. On the other hand, three of the four groups afford striking illustration of the tendency noted elsewhere (Lanford, '39) for a rapid gain in weight to be associated in the same individual with a relatively large gain per 1000 kilocalories. Likewise, the high degree of negative correlation found here between gain in weight and percentage of calcium in the body confirms the same finding in the orange juice experiment, and related observations (Lanford, Campbell and Sherman, '41) on animals during the period of rapid growth.

SUMMARY

Administration of a mixture of citric acid and potassium citrates, in approximately the proportions and quantities which would be supplied by 10 cc. of fresh orange juice daily, increased the rate of growth and the total calcium retention of young rats on a wheat-and-milk diet, but, unlike the orange juice tested earlier in a similar way, had no appreciable effect on the percentage of the dietary calcium which was retained in the body of the growing animal. It thus appears that orange juice favors calcium assimilation in growth through some property in addition to its content of citric acid and citrates and its preponderance of base-forming elements.

The assistance of Grace Guldin Stroup in these studies is gratefully acknowledged.

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THE STATE OF VITAMIN A IN THE LIVER OF THE RAT

II. THE EFFECT OF FEEDING THE VITAMIN OVER EXTENDED PERIODS [1]

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THREE FIGURES

(Received for publication September 30, 1941)

Previous work (Gray, Hickman, and Brown, '40) has shown that no matter in what form vitamin A may be fed to the rat, it appears in the liver esterified in a more or less specific manner. However, the breadth of the elimination curve of this vitamin A present in liver shows that several esters must be present. In that work, high doses of the vitamin were fed over short periods of time. As that experiment represented an extreme case, it seemed desirable to investigate the liver when the rats had been fed more nearly normal doses over extended periods.

Four groups of rats of the Sprague-Dawley strain, containing four to six animals per group, were therefore placed at weaning on the Sherman B diet (Sherman and Campbell, '24) which was supplemented with 500 units of vitamin A daily.[3] The four groups were fed dogfish liver oil, dogfish liver oil

[1] This paper was read before the Biological Section of the American Chemical Society at the 101st meeting, April 9, 1941.

[2] Communication No. 23 from the Laboratories of Distillation Products, Inc.

[3] Elizabeth F. Brown of the Department of Zoology, University of Rochester, supervised the care and feeding of the rats. This work was part of an experiment to determine the toxicity of vitamin A, the results of which are to be published elsewhere. The Sherman B diet is composed of 66% whole wheat, 33% whole milk powder, and 1% sodium chloride.

concentrate, shark liver oil, and shark liver oil concentrate, the concentrates being prepared by distillation of the respective oils. The potencies of the oils in U.S.P. units were: Dogfish liver oil, 16,000; dogfish liver oil concentrate, 236,000; shark liver oil, 105,000; shark liver oil concentrate, 619,000.

Each oil was diluted with cottonseed oil [4] so that a drop contained 100 U.S.P. units of vitamin A. Dosing was started at weaning with 1 drop per day, and the dose gradually increased to 5 drops daily. The feeding period extended through 112 days, each rat receiving a total of 52,000 units.

At the conclusion of the feeding period the animals were sacrificed and the livers removed and immediately placed at —50° C., at which temperature they were kept until used. For examination the livers were thawed, ground with anhydrous sodium sulphate and extracted in a Soxhlet apparatus first with 95% alcohol, then with ether. The solvents were removed under nitrogen and potencies and spectra of the extracts determined. The potencies by the antimony trichloride reaction were determined on an Evelyn photoelectric colorimeter calibrated in U.S.P. units with a vitamin A ester concentrate of known extinction coefficient, using the conversion factor 2110 to convert $E^{1\%}_{1\,\text{cm.}}$ (328 mu) to U.S.P. units. Ultraviolet absorption spectra were determined in cyclohexane solution on a Hilger medium quartz spectograph equipped with a Spekker photometer. Potencies as determined colorimetrically and from the extinction coefficient at 328 mu, using the conversion factor 2110, showed a maximum deviation of 9%. Table 1 gives a summary of these determinations. The recovery of the vitamin is in line with that obtained in the previous work. Distilled shark liver oil concentrate representing a recovery of 72% is, however, definitely higher than the others, but since it represents a single experiment, too much weight cannot be attached to this figure.

The extracts were taken up in ether and washed with aqueous alkali and water. At this point emulsions extremely difficult to deal with were encountered and some mechanical

[4] Wesson.

TABLE 1

The recovery of vitamin A from the liver of the rat after feeding the vitamin over an extended period.

GROUP	NO. OF RATS	SUBSTANCE FED	WEIGHT OF LIVERS		WEIGHT OF EXTRACT	POTENCY OF EXTRACT	TOTAL VITAMIN A FED	TOTAL VITAMIN A REC.	RECOVERY
			Total	Av. Wt. per rat					
			gm.	*gm.*	*gm.*	*I.U. per gm.*	*I.U.*	*I.U.*	*%*
VII	6	Dogfish liver oil	52.5	8.7	3.40	39,000	312,000	133,000	43
IX	4	Dogfish liver oil conc.	38.5	9.6	2.56	34,900	208,000	89,000	43
VI	6	Shark liver oil	52.5	8.7	2.94	60,700	312,000	178,000	57
IV	5	Shark liver oil conc.	46.5	9.3	2.72	68,500	260,000	186,000	72

loss of vitamin occurred. Unfortunately, an accident occurred here resulting in the complete loss of the extract from the dogfish liver oil concentrate, so that no distillation data are available on this group. In the remaining groups the ether solution was dried over anhydrous sodium sulphate, the ether removed, and the residue dissolved in 25 gm. of constant yield oil and 25 gm. of corn oil residue[5] and subjected to analytical

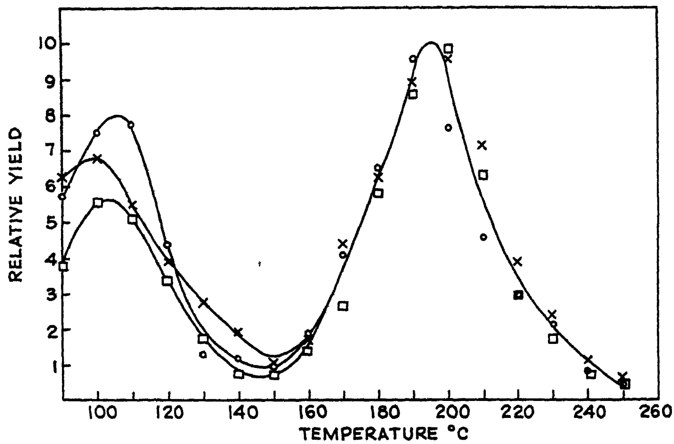

Fig. 1 Vitamin A elimination curves of liver extracts after feeding the vitamin over an extended period:
○ from rats fed dogfish liver oil
× from rats fed shark liver oil
□ from rats fed shark liver oil concentrate

molecular distillation (Hickman, '37). The results of the distillations are plotted in figure 1. The three curves are plotted together, since examination of the individual curves showed that all three were identical within the limits of error of the method. Inspection of figure 1 shows a major maximum at 195° C. with a somewhat smaller maximum at 105° C. Ultraviolet absorption curves were determined on the fractions distilling at each maximum and a representative curve for each is shown in figures 2 and 3. Figure 2 is the absorption curve

[5] A high-boiling fraction, free from vitamin A, obtained from the complete distillation of corn oil.

of the material distilling at 195° C., and is the type of curve obtained for a good quality fish liver oil or concentrate. The absorption curve obtained from fractions distilling at the 105° C. maximum, shown in figure 3, is that of cyclized vitamin A (Castle, Gillam, Heilbron, and Thompson, '34; Embree, '39).

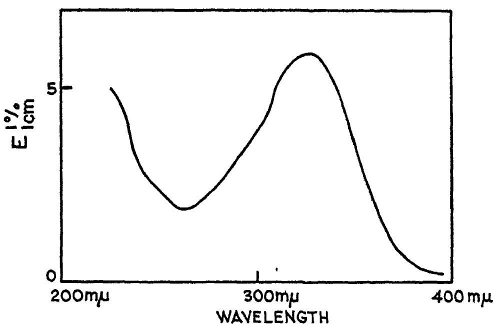

Fig. 2 Typical absorption spectrum of the fraction distilling at 195° C.

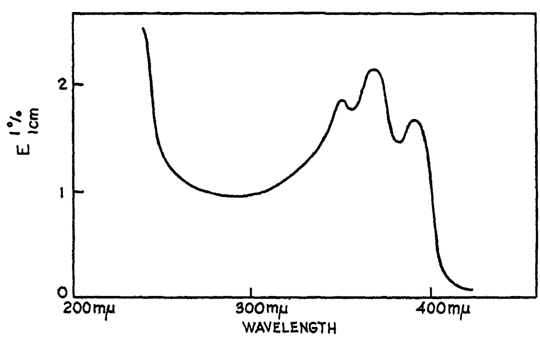

Fig. 3 Typical absorption spectrum of the fraction distilling at 105° C.

DISCUSSION

The complexity of the ester fraction obtained in the short-term feeding experiment is to be noted in comparison with the present experiments. Here the ester fraction is obviously that of a single substance, possibly the palmitate. This transition of vitamin A in the liver from the state of esterification with many fatty acids to that of one fatty acid takes place

gradually. When the vitamin is fed for a period of 60 days, the curve is intermediate in position, showing the presence of more than one ester, but not nearly so complex as when the animal is suddenly flooded with the vitamin (Morgareidge, Gray, and Cawley, unpublished work). Thus there is a selective process going on in the animal which finally results in the esterification of the vitamin with a single fatty acid. Furthermore, this process is extremely slow, taking well over 60 days for completion when the vitamin is fed at a level of 500 units daily. Thus the rat has a definite limit as regards his normal capacity for taking care of vitamin A. Experiments to determine toxicity, and employing much higher levels of the vitamin, bear this out.

Since the completion of this work, one of us (J.D.C.) has found that the cyclized vitamin A present in the extracts is an artifact, arising from the use of alcohol in the extraction of the livers. Details of this phenomenon will be reported more fully elsewhere, but it may be stated here that the cyclization occurs only with the vitamin esters, not the free alcohol, and is not due to the presence of anhydrous sodium sulphate or tissue; cyclization takes place if a vitamin A ester, either synthetic or naturally occurring, is merely refluxed with an alcohol. Since all of several pure synthetic esters have shown this reaction to the same degree, the only effect has been to diminish the amount of ester present, without distorting the ester picture.

CONCLUSIONS

1. When vitamin A is fed in moderate amounts over long periods of time, the vitamin A stored in the liver is gradually transformed from its combination with several fatty acids to a union with but one.

2. Cyclized vitamin A is formed from vitamin A esters on refluxing with alcohols.

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THE RATE OF INCREASE OF BLOOD PLASMA ASCORBIC ACID AFTER INGESTION OF ASCORBIC ACID (VITAMIN C)[1]

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ONE FIGURE

(Received for publication October 25, 1941)

In mass studies of vitamin C nutrition it is not always practical to obtain fasting blood samples. This study was undertaken primarily to ascertain how rapidly and to what extent ascorbic acid ingested with breakfast would cause a rise in blood plasma ascorbic acid above the fasting level; and also to determine the effect of different food sources of the vitamin, and other possible factors, in raising the plasma level.

EXPERIMENTAL

Subjects

The subjects were five college women of the following heights and weights: A, 167.6 cm., 60.78 kilo.; B, 160.0 cm., 45.81 kilo.; C, 174.0 cm., 58.97 kilo.; D, 175.9 cm., 71.67 kilo.; E, 163.8 cm., 58.97 kilo.

[1] Published as scientific paper no. 502, College of Agriculture and Agricultural Experiment Station, State College of Washington, Pullman.

Presented in part at the annual meeting, American Institute of Nutrition, Chicago, April, 1941.

This investigation is part of the regional project of the Northwest States on the ascorbic acid metabolism of college students.

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Plan of experiment

The ascorbic acid content of blood plasma was determined on samples obtained just before breakfast which was eaten at 8:00 a.m. Breakfast consisted of buttered whole-wheat toast and coffee, and 50 mg. of ascorbic acid which was taken either in the crystalline form[3] or in an equivalent amount of either strawberries, orange juice, orange sections, or raw cauliflower. Blood samples were then taken at 8:15, 8:30, and each succeeding half-hour up to and including noon. For each subject, determinations were made on three different mornings with each supplement.

Ascorbic acid determinations

From 0.6 to 0.8 ml. of blood was obtained from the finger tip, and duplicate or in some cases triplicate analyses of the plasma were made. Analyses were made by the micro-method of Farmer and Abt ('36), (tables 1 and 2) and in the later part of the study some analyses were by the Evelyn photoelectric colorimetric micro-method of Mindlin and Butler ('38) with the modification suggested by Bessey ('38) for correction for turbidity.

Comparisons were made of ascorbic acid content of plasma of capillary blood obtained at the same time from the ear and from the finger-tip of the same subject and they were found to give comparable values as shown by the following data. The values are in milligrams of ascorbic acid per 100 ml. plasma:

SUBJECTS:	A	B	C	D	E	F
Ear	1.01	1.18	1.09	1.09	0.92, 0.88	0.76
Finger tip	1.05	1.18	1.07	1.11	0.92, 0.86	0.74

In our laboratory the maximum error from possible difficulty in determining the end-point by the micromethod of Farmer and Abt ('36) has been found to be 0.08 mg. per 100 ml. blood plasma so that differences of less than 0.08 mg. are not considered as indicative of change in the plasma value.

[3] We are indebted to Merck and Company, Rahway, New Jersey, for a generous supply of pure ascorbic acid.

Each blood sample was analyzed without delay but studies were also made to determine the extent of possible changes in ascorbic acid content if samples were allowed to stand in the refrigerator at 11° C. for varying lengths of time at any stage in the procedure. Whole blood with an original value of 1.41 mg. ascorbic acid and analyzed after 3¾ hours was found to contain 1.35 mg. per 100 ml. of plasma; this difference is not significant. Other samples of whole blood with initial values of 1.23 and 1.25 mg. after 2 hours' storage showed values of 1.14 and 1.20 mg. respectively. Samples of whole blood which were centrifuged and stored with the plasma remaining in contact with the corpuscles showed no change in ascorbic acid content when analyzed after 2, 3, and 4 hours but one sample with an initial value of 0.92 mg. after 6½ hours' storage showed a value of 0.71 mg., a loss of 23%. Similarly, some ten different samples of deproteinized plasma stored in the refrigerator at 11° C. for from 2 to 5 hours showed no appreciable change in ascorbic acid content.

The ascorbic acid contents of the fruits and the cauliflower were determined by the micromethod using the Evelyn photoelectric colorimeter (Bessey, '38). The average ascorbic acid values obtained were: orange juice 0.49 mg. per milliliter (fourteen samples); orange flesh 0.49 mg. per gram (ten samples); strawberries 0.64 mg. per gram (eighteen samples); and cauliflower 0.50 mg. per gram (five samples; only the florets of the cauliflower were used).

RESULTS

Influence of ingested ascorbic acid on blood plasma level

When ascorbic acid was taken in the crystalline form at 8:00 a.m. the blood plasma level showed a slight increase by 8:30 a.m. in some cases, and a definite increase by 9:00 a.m. for all subjects, attaining a maximum by 9:30 and returning to the original fasting level by 11:00 or 11:30 a.m. The same subjects consistently showed the same general picture of absorption rate on each of the three different mornings that

TABLE 1

Ascorbic acid content of blood plasma after ingestion of 50 mg. ascorbic acid or an equivalent amount from orange juice and strawberries.

SUBJECT	SUPPLEMENT	MG. ASCORBIC ACID PER 100 ML. PLASMA AT GIVEN HOUR IN A.M.										MAXIMUM INCREASE IN PLASMA VALUE
		7:59	8:15	8:30	9:00	9:30	10:00	10:30	11:00	11:30	12:00	*mg.*
A	crystalline ascorbic acid	1.38	1.41	1.41	1.56	1.63	1.58	1.50	1.46	1.43	1.41	0.25
		1.44	1.44	1.44	1.52	1.62	1.60	1.52	1.50	1.44	1.44	0.18
		1.29	1.31	1.29	1.43	1.61	1.52	1.38	1.29	1.29	1.26	0.32
	orange juice	1.20	1.20	1.20	1.32	1.36	1.46	1.36	1.28	1.20	1.20	0.26
		1.34	1.34	1.34	1.43	1.64	1.60	1.51	1.43	1.43	1.34	0.30
		1.02	0.97	0.97	1.14	1.32	1.14	1.06	1.06	0.97	0.97	0.30
	strawberries	1.22	1.26	1.26	1.26	1.38	1.51	1.38	1.30	1.26	1.26	0.29
		1.10	1.10	1.10	1.10	1.20	1.34	1.24	1.20	1.10	1.10	0.24
		1.06	1.06	1.06	1.06	1.26	1.40	1.26	1.18	1.10	1.06	0.36
B	crystalline ascorbic acid	1.06	1.04	1.12	1.32	1.36	1.32	1.28	1.14	1.16	1.12	0.30
		1.46	1.41	1.45	1.67	1.70	1.58	1.50	1.41	1.43	1.41	0.24
		1.10	1.10	1.20	1.20	1 38	1.31	1.24	1.10	1.08	1.10	0.28
	orange juice	1.14	1.14	1.23	1.32	1.41	1.32	1.28	1.23	1.18	1.14	0.27
		1.09	1.09	1.09	1.26	1.34	1.22	1.18	1.14	1.09	1.09	0.25
		1.09	1.09	1.09	1.22	1.26	1.30	1.18	1.14	1.09	1.09	0.21
	strawberries	1.34	1.34	1.34	1.34	1.47	1.64	1.51	1.43	1.43	1.38	0.30
		1.06	1.10	1.06	1.06	1.18	1.32	1.23	1.18	1.14	1.10	0.26
		1.23	1.19	1.23	1.32	1.41	1.50	1.41	1.32	1.32	1.23	0.27
C	crystalline ascorbic acid	1.21	1.23	1.23	1.32	1.50	1.41	1.34	1.26	1.23	1.23	0.29
		0.84	0.84	0.92	1.01	1.09	1.01	0.96	0.92	0.84	0.84	0.25
	orange juice	1.00	1.04	1.04	1.28	1.20	1.12	1.10	0.96	0.96	0.96	0.28
		1.09	1.18	1.18	1.26	1.38	1.34	1.26	1.18	1.09	1.01	0.29
		0.97	0.97	1.01	1.06	1.23	1.18	1.06	1.06	1.02	0.97	0.26
	strawberries	1.43	1.43	1.38	1.43	1.51	1.68	1.60	1.51	1.51	1.43	0.25
		1.06	1.06	1.06	1.06	1.18	1.32	1.28	1.14	1.14	1.06	0.26
		1.06	1.06	1.06	1.14	1.23	1.32	1.18	1.14	1.14	1.10	0.26
D	crystalline ascorbic acid	0.92	0.88	0.92	1.10	1.10	1.20	1.04	0.99	0.94	0.90	0.28
		0.94	0.92	0.94	1.04	1.10	1.04	1.02	0.97	0.94	0.97	0.16
		0.88	0.88	0.97	1.08	1.06	1.06	0.97	0.88	0.88	0.90	0.20
	orange juice	1.06	1.06	1.14	1.23	1.23	1.14	1.14	1.06	1.06	1.06	0.17
		1.06	1.10	1.06	1.18	1.32	1.23	1.23	1.14	1.06	1.06	0.26
		1.08	1.06	1.06	1.23	1.32	1.21	1.14	1.14	1.06	1.06	0.24
	strawberries	1.06	1.06	1.06	1.10	1.23	1.36	1.32	1.23	1.14	1.14	0.30
		1.09	1.09	1.09	1.18	1.26	1.30	1.26	1.18	1.14	1.09	0.21
		1.18	1.18	1.18	1.18	1.26	1.38	1.34	1.26	1.18	1.18	0.20

determinations were made; and for four of the subjects of differing height and weight there was close agreement in the rate and time of reaching the maximum of absorption of the ascorbic acid. These data are summarized in table 1. The extent of the increase in plasma value was not always the same for each subject but in all cases the same general trend was observed with increases ranging from 0.16 to 0.32 mg. per 100 ml. blood plasma.

Some foods as a source of ascorbic acid

When either orange juice or sections of whole orange was taken in amounts to supply 50 mg. ascorbic acid, the maximum increases in plasma ascorbic acid levels were comparable to those obtained when crystalline ascorbic acid was ingested and the time of reaching the peak of increase was also similar (table 1 and fig. 1). When strawberries were the source of ascorbic acid the peak of the curve was reached in 2 hours and when raw cauliflower was taken, the time of maximum increase in plasma values was delayed until 10:30 a.m. with return to the original fasting level by 11:30 a.m. (fig. 1). Those fruits or vegetable which have considerable cellular material and therefore are more slowly digested were thus found to give a delayed response in blood plasma increase.

Influence of iron salts

When iron salts, in the form of 6 grains of ferrous sulphate,[4] or an equivalent amount of iron as ferric ammonium citrate were taken at the same time as the 50 mg. dose of ascorbic acid, the increase in plasma ascorbic acid was apparently uninfluenced for two subjects and was somewhat delayed for one subject. A typical curve for one subject is shown in figure 1.

Farmer, Abt and Aron ('40) reported that 6 grains or more of ferrous sulphate caused a sharp drop in plasma ascorbic acid level; however the iron salts were given over a period of 20 days and doses smaller than 6 grains daily were found to be ineffective.

[4] Feosol.

Influence of high fat intake

In order to determine whether a high intake of fat such as might be taken at breakfast would affect the plasma level, 50 gm. of butter were eaten with the standard breakfast at 8:00 a.m. This amount was calculated to be a possible intake if eggs, bacon, cream and butter were eaten at breakfast. A typical curve for one subject is shown in figure 1; subjects A

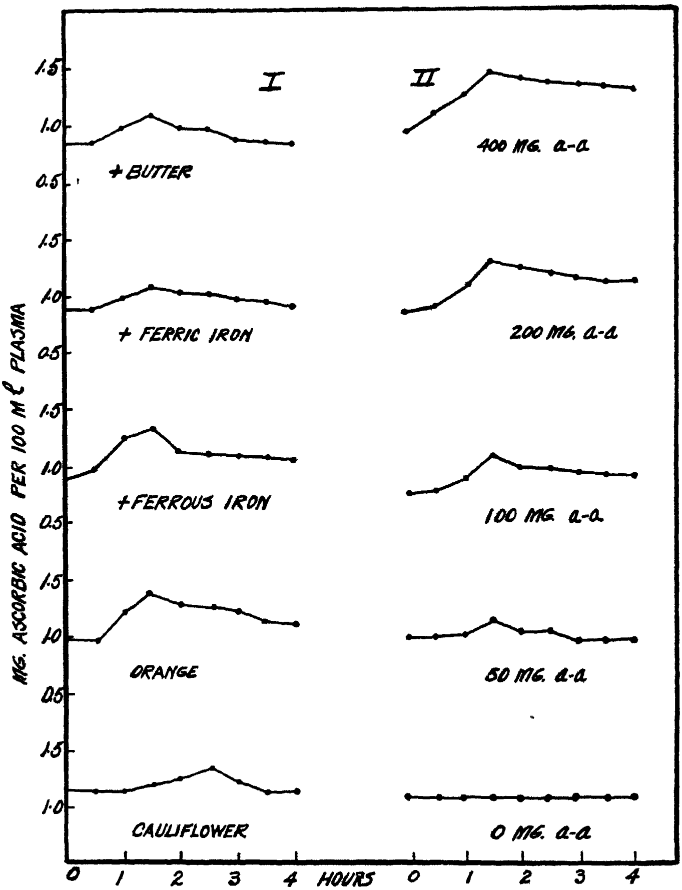

Fig. 1 Typical curves showing plasma content of ascorbic acid; I, after in gestion of 50 mg. of the vitamin as cauliflower or orange, or in the crystalline form plus butter, or plus ferrous or ferric iron; II, one subject with varying intakes of crystalline ascorbic acid.

and B showed the same general trend as previously noted with the peak of the curve being reached at 9:30 a.m., and for subject D the peak was attained at 10:30 a.m.

Influence of fasting level of plasma ascorbic acid on the extent of increase in response to a 50 mg. intake

The original fasting level may be expected to influence the extent to which the plasma ascorbic acid will be increased, but not the time at which the peak of the rise is reached. This was studied for subject D and the data are summarized in table 2. When the fasting level was 1.4 mg. per 100 ml. plasma, the ingestion of 50 mg. crystalline ascorbic acid caused an increase by 9:30 a.m. of from 0.33 to 0.42 mg. However, at a later date when the fasting level had been dropped to 0.8 mg. per 100 ml. of plasma the ingestion of 50 mg. ascorbic acid caused an increase of from 0.16 to 0.28 mg. per 100 ml. The fasting level was again lowered by going on a diet from which all fruits and vegetables were excluded; in 15 days the plasma content dropped from a level of 1.28 mg. to 0.48 mg. per 100 ml. At this level the ingestion of 50 mg. ascorbic acid failed to produce any significant change in plasma ascorbic acid on 3 successive days. Therefore, on each of the next 3 days 150 mg. crystalline ascorbic acid was ingested and a significant increase was obtained by 9:30 a.m. each day.

Influence of amount of ascorbic acid ingested

Subject E was followed through a series of determinations in which the fasting level was approximately the same but the amount ingested was varied as 0, 50, 100, 200 and 400 mg. crystalline ascorbic acid. Typical curves for this subject at each level of intake are shown in figure 1. With no intake of ascorbic acid there was no change in the ascorbic acid content of the blood plasma; a similar finding was reported by Taylor, Chase and Faulkner ('36). As the intake of ascorbic acid was increased, the plasma content of this substance increased more rapidly and reached a correspondingly greater maximum until

TABLE 2

Ascorbic acid content of blood plasma of one subject with different initial levels and intake.

DATE	SUPPLEMENT	MG. ASCORBIC ACID PER 100 ML. PLASMA AT GIVEN HOUR IN A.M.										MAXIMUM INCREASE
		8:00	8:15	8:30	9:00	9:30	10:00	10:30	11:00	11:30	12:00	
												mg.
Nov. 15 '39	50 mg. ascorbic acid [1]	1.39	—	—	1.43	1.66	1.81	1.70	1.68	1.66	1.45	0.42
Nov. 16		1.43	—	—	1.51	1.68	1.68	1.76	1.68	—	1.51	0.33
Nov. 17		1.41	—	—	1.45	1.64	1.68	1.76	1.68	1.60	1.51	0.35
Feb. 19 '40	50 mg. ascorbic acid	0.92	0.88	0.92	1.10	1.10	1.20	1.04	0.99	0.94	0.90	0.28
Feb. 20		0.94	0.92	0.94	1.04	1.10	1.04	1.02	0.97	0.94	0.97	0.16
Feb. 21		0.88	0.88	0.97	1.08	1.06	1.06	0.97	0.88	0.88	0.90	0.20
April 1 '40	No fruits or vegetables	1.28										
April 10		0.72										
April 12		0.59										
April 15	50 mg. ascorbic acid	0.48	0.46	0.44	0.44	0.48	0.44	0.44	0.44	0.44	0.40	—
April 16		0.35	0.38	0.35	0.38	0.44	0.35	0.35	0.35	0.35	0.35	—
April 17		0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	—
April 18	150 mg. ascorbic acid	0.35	0.35	0.35	0.53	0.62	0.53	0.44	0.46	0.44	0.44	0.27
April 19		0.44	0.44	0.44	0.44	0.53	0.58	0.53	0.53	0.44	0.44	0.14
April 20		0.44	0.44	0.44	0.44	0.55	0.53	0.48	0.44	0.44	0.46	0.11

[1] Supplement taken at 8:15.

with intakes of 200 and 400 mg. of ascorbic acid the plasma level failed to return to the original fasting level within 4 hours.

DISCUSSION

The data summarized in table 1 and figure 1 show the rapidity with which ascorbic acid is absorbed and appears in the blood stream; they also show that when the intake is 50 mg. ascorbic acid, that is, an amount which might normally be taken at breakfast in a serving of fruit or in a small glass of orange juice, the blood plasma returns to the original fasting level in 3 to 4 hours after the meal. Alt, Chinn and Farmer ('39) have similarly reported that for six normal subjects, with an intake of 75 to 100 mg. ascorbic acid as orange juice taken at breakfast, the plasma ascorbic acid returned to the original fasting level 3 hours after the meal. Even when the intake is high, as with amounts of 400 mg. ascorbic acid, the plasma concentration tends to return to the original fasting level. When it is not practicable to obtain blood samples prior to breakfast, samples may be taken in the morning if no fruit or other source of ascorbic acid has been consumed within 4 or 5 hours.

Some investigators have questioned the value of plasma ascorbic acid as a measure of nutritional status because the values fall much more rapidly in the plasma than in the white cells (Crandon, Lund and Dill, '40; Butler and Cushman, '40) and because plasma values of practically zero have been obtained without any obvious signs of scurvy. However, the data obtained in the present study indicate that the fasting plasma level tends to remain constant from day to day with a diet of approximately the same intake, and when vitamin C-containing foods are withdrawn from the diet (table 2) there is a decrease in the plasma ascorbic acid content. The ascorbic acid content of the plasma reflects the dietary intake of this vitamin and although low plasma concentration is apparently not an indication of subclinical scurvy, these findings are believed to be indicative of the value of plasma determinations

as a measure of the state of vitamin C nutrition particularly for group studies. Recently Bryan and co-workers ('41) have also offered evidence for the close relation between dietary intake and plasma concentration of ascorbic acid.

SUMMARY

Five subjects, studied for rate of increase in plasma ascorbic acid after ingestion of 50 mg. of this vitamin, showed close similarity in response for the different subjects and for the same subject at different times.

The ascorbic acid content of blood plasma began to rise within 30 to 60 minutes after ingestion of 50 mg. of the vitamin either in crystalline form or from fruits, and returned to the fasting level in 3 to 4 hours.

The maximum increase in plasma level of ascorbic acid was reached within 1½ hours after ingestion of 50 mg. of this substance in either the crystalline form, as orange juice, or as orange sections; in 2 hours when strawberries were the source of ascorbic acid; and in 2½ hours when cauliflower was eaten.

The curve for increase of plasma ascorbic acid was not influenced by fat or by ferrous or ferric salt taken at the same time as the ascorbic acid.

When the intake was raised to 400 mg. ascorbic acid there was a greater increase in plasma concentration and a longer time elapsed before returning to the original fasting level.

Ingestion of 50 mg. ascorbic acid did not cause an increase in its plasma concentration when the fasting level was 0.5 mg. or less per 100 ml. plasma.

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Conservation of Scholarly Journals

The American Library Association created this last year the Committee on Aid to Libraries in War Areas, headed by John R. Russell, the Librarian of the University of Rochester. The Committee is faced with numerous serious problems and hopes that American scholars and scientists will be of considerable aid in the solution of one of these problems.

One of the most difficult tasks in library reconstruction after the first World War was that of completing foreign institutional sets of American scholarly, scientific, and technical periodicals. The attempt to avoid a duplication of that situation is now the concern of the Committee.

Many sets of journals will be broken by the financial inability of the institutions to renew subscriptions. As far as possible they will be completed from a stock of periodicals being purchased by the Committee. Many more will have been broken through mail difficulties and loss of shipments, while still other sets will have disappeared in the destruction of libraries. The size of the eventual demand is impossible to estimate, but requests received by the Committee already give evidence that it will be enormous.

With an imminent paper shortage attempts are being made to collect old periodicals for pulp. Fearing this possible reduction in the already limited supply of scholarly and scientific journals, the Committee hopes to enlist the cooperation of subscribers to this journal in preventing the sacrifice of this type of material to the pulp demand. It is scarcely necessary to mention the appreciation of foreign institutions and scholars for this activity.

Questions concerning the project or concerning the value of particular periodicals to the project should be directed to Wayne M. Hartwell, Executive Assistant to the Committee on Aid to Libraries in War Areas, Rush Rhees Library, University of Rochester, Rochester, New York.

CONGENITAL MALFORMATIONS INDUCED IN RATS BY MATERNAL NUTRITIONAL DEFICIENCY

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ONE PLATE (FOUR FIGURES)

(Received for publication October 29, 1941)

In previous communications ('40, '41) we reported that congenital malformations appeared in the offspring of externally normal albino rats of the Sprague-Dawley strain when the females were reared and bred on a deficient diet. On external inspection the defective offspring frequently showed shortening of the mandible and protrusion of the tongue, shortening and distortion of the extremities and various degrees of syndactylism of the hands and feet; thinness of the abdominal wall in the ventral midline, protrusion of abdominal viscera, reduction in size of the uro-genital papilla and defects of the eyes also were observed in rare cases. After the specimens were cleared by the Schultze-Dawson method it was found that certain bones were often abnormal while other bones apparently were never affected. Thus, in spite of great variations, a certain pattern of skeletal malformations was recognized in the offspring of the females bred on this deficient diet. This pattern which has been described in detail previously (Warkany and Nelson, '41) will be referred to as "pattern of diet I" in the following paragraphs. Figures 1 and 2 illustrate an abnormal animal in its external appearance and an abnormal cleared specimen respectively. In the present publication we wish to present further data on the nutritional aspects of the experiments.

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Breeding results on diet I

The deficient diet referred to is essentially the Steenbock and Black diet no. 2965 ('25) supplemented with viosterol to forestall the development of rickets. This diet which will be referred to as "diet I" in the following pages had the following percentage composition: whole yellow corn meal[1] 76, wheat gluten[2] 20, calcium carbonate C.P. 3 and sodium chloride C.P. 1. These ingredients were mixed thoroughly and fed in cups ad libitum. Sixty International Units of vitamin D as viosterol were administered to each female by pipette every tenth day. The females were put on diet I at the age of 4 weeks. They grew slowly and the process of maturation was delayed. However, at the age of 5–6 months they frequently attained a weight of 150 gm. and regular cycles appeared. Such females were bred to males of the same strain that had been reared on the adequate stock diet. When sperm was found in the vaginal smear the females were isolated. The vaginal smears were then continued to make certain the diagnosis of pregnancy which was considered established with the appearance of the blood sign. During the period of expected parturition the pregnant females were watched day and night in order to prevent the mothers from eating their young during or after delivery.

In fifty-nine females on diet I, 122 pregnancies were observed. Ninety-three of these pregnancies were carried through and resulted in the delivery of 484 offspring. These consisted of 295 normal and 189 abnormal young. Of the ninety-three litters observed in this group forty-three litters consisted entirely of normal offspring, thirty-three entirely of abnormal and seventeen of both normal and abnormal offspring. The average litter size on this diet was only 5.20. Abnormal young and fetuses in resorption were frequently found in the same pregnancy (figs. 3 and 4). Of 122 pregnancies twenty-nine were entirely resorbed. This incidence of

[1] Bolted, Tuxedo brand.

[2] Pure Gluten Food Company.

resorptions in females on diet I is very high compared with that found in females on adequate diets. It appears that abnormal fetuses are frequently resorbed before the pregnancy is finished. Of the fifty-nine females on diet I thirty-two were pregnant more than once. The results of these repeated (two to six) pregnancies indicate that in later pregnancies abnormal litters occur more frequently. Eventually all the females on this diet gave birth to one or more abnormal litters, with the exception of two animals which have been bred five times and have as yet not delivered abnormal offspring.

Breeding results on a stock diet

A group of thirty-eight females of the same strain were reared and bred on Bills' modification of Steenbock's stock diet (Bills et al., '28), which had the following percentage composition: yellow corn meal 57, whole milk powder 25, linseed oil meal 12, crude casein 3.7, alfalfa leaf meal 1.5, iodized table salt 0.4, and calcium carbonate 0.4. Ninety-one pregnancies were observed in this group. Only one of these ended in resorption and ninety litters were obtained. These consisted of 614 young, of which 613 were normal and one was abnormal. The latter had a shortened humerus and femur but normal tibia, mandible and ribs; this pattern of abnormalities differs from the pattern of diet I. The average litter size on the stock diet was 6.82.

Breeding results on a modification of diet I with $CaCO_3$ content reduced and 2% liver added (diet II)

While the breeding experiments on diet I were proceeding, twenty-three females of the Sprague-Dawley strain were reared and bred on a diet consisting of: yellow corn meal 78, wheat gluten 18, calcium carbonate C.P. 1, sodium chloride 1 and dried pig liver 2%, a diet devised by Remington ('37) as an "improved goitrogenic diet." In our experiments viosterol was administered to the animals fed this diet as it was to

those fed diet I. The dried pig liver was prepared as recommended by Remington. On this diet fifty-five pregnancies resulting in fifty-three litters were observed. They consisted of 350 young, of which 348 were normal and two abnormal. The two abnormals were not of the pattern of diet I. One animal was dwarfed and tailless, and after clearing showed a fused sternum but no other skeletal defects. The other abnormal young was of normal size and externally the mouth appeared reduced in size, the upper lip showed a double cleft and the tongue did not protrude. After clearing, fusion of both halves of the mandible in the midline and a bifurcation of a rib on each side were seen. The average litter size on diet II was 6.60. Two pregnancies ended in resorption.

Breeding results on a modification of diet I with $CaCO_3$ content reduced and 0.5% liver added (diet VII)

This diet consisted of yellow corn meal 78, wheat gluten 19.5, sodium chloride C.P. 1, calcium carbonate C.P. 1, and dried pig liver 0.5%. Vitamin D was given as in diet I. Forty pregnancies were observed in fourteen females on this diet. They resulted in thirty-seven litters consisting of 236 young. Of these 233 were normal and three abnormal. The three abnormal animals were of the pattern of diet I. The average litter size was 6.37. Three pregnancies ended in resorption.

While the females on diet I had 39.05% abnormal offspring, those on diet II had none and those on diet VII had only 1.27% abnormal offspring. Diet II and diet VII differ from diet I essentially in two respects, the addition of liver and the reduction of calcium carbonate. It was thought advisable to test the preventive power of these two factors separately. Therefore two additional diets were devised: Diet VI which is essentially identical with diet I except for the addition of 2% liver, and diet V which is essentially identical with diet I except for the reduction of calcium carbonate from 3% to 1%.

Breeding results on a modification of diet I with 2% liver added (diet VI)

Nineteen females were reared and bred on diet VI which had the following percentage composition: yellow corn meal 76, wheat gluten 18, calcium carbonate C.P. 3, sodium chloride C.P. 1, and pig liver 2. Viosterol was given as in diet I. Fifty-six pregnancies and fifty-one litters were observed in the females on this diet. The offspring consisted of 322 normal and no abnormal young. The average litter size was 6.31. Five pregnancies ended in resorption.

Breeding results on a modification of diet I with $CaCO_3$ content reduced (diet V)

Twenty-four females were reared and bred on diet V which had the following percentage composition: yellow corn meal 78, wheat gluten 20, calcium carbonate C.P. 1, and sodium chloride C.P. 1. Viosterol was given as with diet I. Sixty pregnancies resulting in fifty-five litters occurred in this group. Three hundred and forty-nine young, of which 316 were normal and thirty-three were abnormal, were obtained on this diet. The abnormalities seen externally and after clearing showed the pattern of diet I. The average litter size was 6.35. Five pregnancies ended in resorption.

Breeding results on a modification of diet I with alcoholic liver extract added (diet IV)

An alcoholic extract of liver was made and added to diet I in order to test its preventive capacity.

Dried pig liver as prepared for diet II was extracted with 95% alcohol in a Soxhlet apparatus for 3 days. An amount of extract equivalent to 4 gm. of dried liver was used in making up 100 gm. of this diet. The extract was added to the corn meal and the alcohol evaporated on a steam table.

Ten females on this diet had thirty-seven pregnancies and thirty-five litters. Two hundred and thirty-five young, all normal, were obtained. The average litter size was 6.71. Two pregnancies ended in resorption.

Table 1 summarizes the breeding results on all the diets discussed in this communication. Only abnormal offspring of the pattern of diet I are listed as "abnormal" in this table.

The occurrence of abnormal offspring of the pattern of diet I was prevented when the females were fed diet I supplemented by 2% liver or alcoholic liver extract. A supplement of 0.5% liver did not prevent the abnormalities entirely but reduced their incidence materially. Reduction of the calcium content of diet I also resulted in a reduced incidence of abnormalities.

TABLE 1

Breeding results on different diets.

MOTHER REARED ON	OFFSPRING			ABNORMAL OFFSPRING PER CENT OF TOTAL OFFSPRING
	Total	Normal	Abnormal	
Diet I	484	295	189	39.05
Diet I with $CaCO_3$ content reduced (diet V)	349	316	33	9.46
Diet I with $CaCO_3$ content reduced and 0.5% liver added (diet VII)	236	233	3	1.27
Diet I with $CaCO_3$ content reduced and 2% liver added (diet II)	348	348	0	0
Diet I with 2% liver added (diet VI)	322	322	0	0
Diet I with alcoholic liver extract added (diet IV)	235	235	0	0
Stock diet	613	613	0	0

Breeding results on alternating diets

It seemed of interest to find out whether a female which had been on diet I and had had abnormal offspring would have normal offspring when changed to a liver-containing diet before her next mating and conversely, whether a female which

had had normal offspring while on a liver-containing diet would have abnormal offspring when changed to diet I. The following is a representative experiment of this type.

Female 45 B, reared and bred on diet I, was mated to male 47 R and delivered abnormal offspring. On the day of this delivery she was changed to diet II and then mated to the same male. In the subsequent litter she delivered entirely normal offspring. The breeding on alternating diets was repeated several times, the change always being made on the day of delivery and before the next mating. The results are presented in table 2. In this experiment the same female and

TABLE 2

Breeding results on alternating diets.

ON DIET	OFFSPRING Normal	Abnormal
I	0	4
II	8	0
I	6	1
II	10	0
I	8	2
I	0	7
II	3	0

the same male were mated seven times. As may be seen from the table, alternating the dietary regime of the mother resulted in the production of alternate abnormal and normal litters. In many similar breeding experiments on alternating diets it was found that a change from diet I to the liver-containing diet invariably resulted in normal offspring. However, the change from the liver-containing diet to diet I was sometimes not followed immediately by the appearance of an abnormal litter and only when the females were kept on diet I during subsequent pregnancies did abnormal offspring appear.

Breeding results on diet I obtained with a different strain of rats

The results so far reported were obtained with albino rats of the Sprague-Dawley strain. Through the kindness of Dr. L.

Emmett Holt of the Pediatric Department of Johns Hopkins University we were supplied with a different strain of rats which had been inbred there for many years. This strain was derived originally from Dr. E. V. McCollum's rat colony and it consisted of albino and yellow and black hooded rats.

These rats were put on diet I at the age of 1 or 2 months and bred to males of the same strain as soon as regular cycles were observed. Twelve females had eighteen litters consisting of 123 young of which 48, or 39.02%, were abnormal. Thus the incidence of abnormalities in this strain was equal to that observed in the Sprague-Dawley strain. The congenital defects of the offspring resembled in all respects those described as the pattern of diet I in the Sprague-Dawley strain. Abnormalities were found in albino and in hooded offspring.

DISCUSSION

It has been asserted often that faulty nutrition of the fetus may be a cause of congenital malformations. The faulty nutrition may have its origin in various disturbances. Faulty implantation of the ovum, diseases of the placenta, interruption of the umbilical blood flow, toxic substances transmitted through the placenta and deficiencies in the diet of the mother have been suggested as causes of deformities in the mammalian embryo or fetus. The last-mentioned cause has aroused new interest in recent years. When it was recognized that minute amounts of certain foodstuffs were required for satisfactory nutrition a qualitative malnutrition of the pregnant animal could be suspected to be the cause of a faulty development of the embryo.

Hale ('35) described microphthalmia, cleft palate and other congenital malformations in the offspring of vitamin A-deficient sows. Andersen ('41) reported a high incidence of congenital diaphragmatic hernia in the young of rats bred on a diet deficient in vitamin A. However, Hart and coworkers ('33), Hughes ('34), and Cannon ('40) found no congenital malformations in the offspring of vitamin A-deficient animals.

Moore and coworkers ('35) observed blindness that developed during the intra-uterine period and was associated with a constriction of the optic nerve in calves whose dams had received rations with a poor quality roughage. Micromelia of chicken embryos caused by a nutritional deficiency of the laying hens was reported by Byerly and coworkers ('35).

In our experiments a significant difference was found in the incidence of congenital abnormalities in newborn rats according to the diet of the mother. More than one-third of the offspring of females fed diet I showed skeletal abnormalities of a definite pattern. This pattern did not occur in the offspring of females fed a balanced stock diet. This stock diet differed from diet I in so many respects that it was not possible to draw conclusions as to the identity of the protective factor in the stock diet. Abnormalities of the pattern of diet I were also absent in the offspring of the females bred on diet II. This ration differed from diet I in two respects only — in the addition of 2% pig liver and in the reduction of the calcium carbonate content from 3 to 1%. In order to decide which of these two changed factors prevented the abnormalities, diet VI was devised, differing from diet I only in that 2% liver was added. Since the offspring of the females reared on this diet were also entirely normal, it may be concluded that liver contains a factor which prevents the appearance of the abnormalities of the pattern of diet I. The nature of this preventive factor has not yet been established. However, it seems to be present in pig liver in large amounts, since the addition of 2% of the dried organ proved effective. That the preventive factor in liver can be extracted by alcohol is shown by the results observed with diet IV with which entirely normal offspring were obtained (table 2). The offspring of females bred on diet VII which contained only 0.5% dried liver were not entirely protected. Three of the 236 offspring obtained on this diet showed abnormalities of the pattern of diet I.

The high calcium content of diet I apparently contributed in some way to the high incidence of congenital abnormalities seen in the offspring of females bred on this diet. On diet V

which differs from diet I in only one respect, the reduction of calcium carbonate from 3 to 1%, a significantly lower incidence of congenital malformations was observed.

All of these experimental results are in accord with our assumption that the congenital malformations described are due to a faulty diet of the mother. This assumption is further supported by the observation that females on diet I which had normal offspring in the first litter frequently had abnormal offspring in the second or subsequent litters. It is possible that a store of the protective factor present in the mother at the beginning of the breeding experiment becomes exhausted during one or more pregnancies. The experiments with alternating diets lend further support to the explanation of the malformations on a nutritional basis. When a female which had had abnormal offspring on diet I was changed to the liver-containing diet II and bred again, she always produced entirely normal offspring. The preventive factor apparently becomes effective very rapidly. However, when a female which had had normal offspring on diet II was changed to diet I and was bred again, she had sometimes normal and sometimes abnormal offspring. This behavior can be explained by the assumption that in the first case (normal offspring after changing to diet I) the female had stored a reserve of the preventive factor adequate for this pregnancy, while in the second case (abnormal offspring after changing to diet I) the storage was inadequate.

The fact that identical abnormalities could be obtained under similar dietary conditions in different strains of rats points also to a nutritional basis for the phenomenon described. The experiments reported have given rise to several questions which are under investigation at the present time. An attempt is being made to identify the protective factor in liver or its alcoholic extract. At the same time experiments are being conducted in which various known accessory foodstuffs are being added to diet I in order to test their protective value. An investigation concerned with the critical period of

development in which the abnormalities are determined is also in progress.

SUMMARY

1. Skeletal abnormalities occurred in about one-third of the offspring of female rats reared and bred on Steenbock and Black's rachitogenic diet no. 2965 supplemented with viosterol (diet I).

2. Similar skeletal defects were not found in the offspring of females of the same strain reared and bred on a stock diet.

3. Similarly no such defects were observed in the offspring of females of the same strain reared and bred on diet II, a diet which differs from diet I in that it contains 2% dried pig liver and only 1% of calcium carbonate.

4. The defects were also absent in the offspring of females reared and bred on diet I supplemented by 2% pig liver only (diet VI).

5. If in diet I the calcium carbonate content was reduced from 3 to 1% only about one-tenth of the offspring showed abnormalities of the pattern of diet I.

6. By alternately breeding the same female on diets I and II, abnormal and normal litters could be produced alternately.

7. The same pattern of abnormalities was obtained in the offspring of rats of two different strains reared and bred on diet I.

8. Apparently a nutritional factor that is absent or inadequate in diet I and present in liver in large amounts is necessary for the normal intra-uterine development of the rat.

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PLATE 1

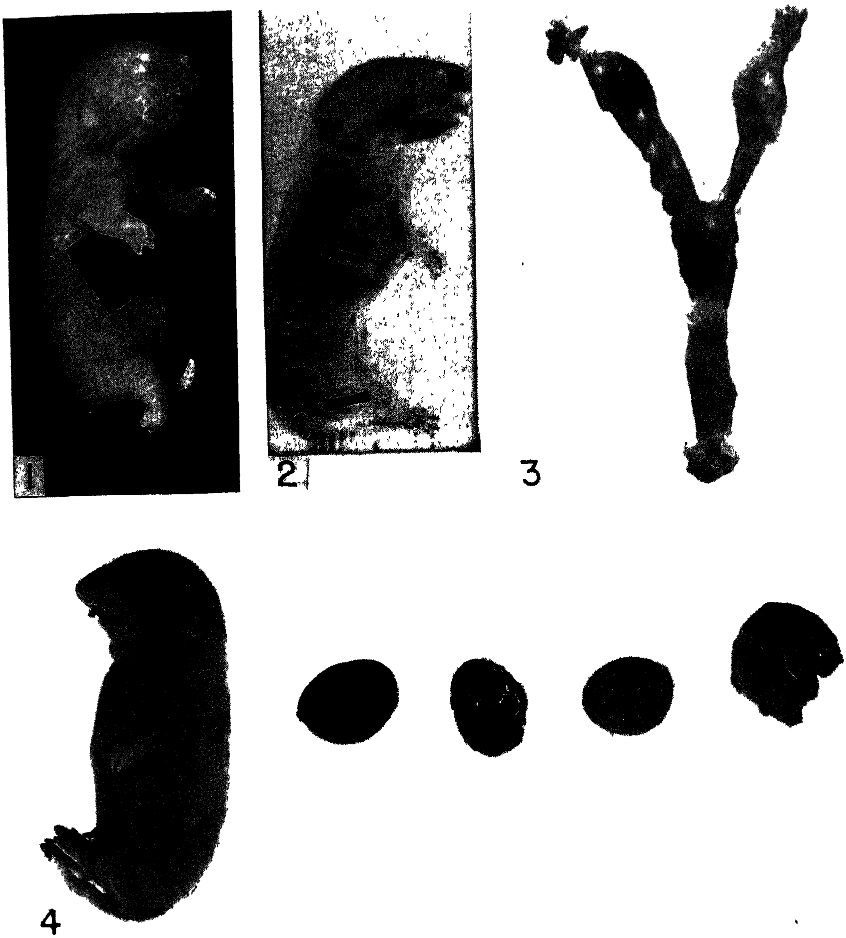
EXPLANATION OF FIGURES

1 Abnormal newborn rat showing receding mandible, protruding tongue, syndactylism of hand and deformed leg.

2 Cleared specimen of an abnormal newborn rat showing shortening of the mandible, absence of the radius, great reduction in size of the ulna, fusion of the ribs, absence of the tibia and reduction in size of the fibula.

3 Ventral view of reproductive tract of a parturient rat reared and bred on diet I. From the left uterine horn an abnormal young had been removed. In the right uterine horn and in the region of the cervix fetuses in resorption may be seen. Several additional implantation sites may be noted in the right uterine horn.

4 A litter of one abnormal young and four fetuses in absorption.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THE EFFECT OF THE LEVEL OF FAT IN THE DIET UPON UTILIZATION OF VITAMIN A [1]

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This is a report on the second phase of an experiment planned to study the effect of various dietary factors upon utilization of vitamin A. Results reported in a previous paper (Muelder and Kelly, '41) indicated an effect of restricted intake of basal ration upon utilization of vitamin A. Because of the restriction in food intake, the calorie-control rats received not only fewer calories but less of all the nutrients (excepting the B vitamins) than did the positive control rats. It seemed probable that the restriction of some particular component of the basal ration may have been a causative factor in the consistently less favorable response to equal levels of vitamin A supplement for the rats on restricted food intake.

In view of the fact that vitamin A is a fat soluble vitamin, the role of fat in the basal diet as it affects the utilization of vitamin A seemed particularly pertinent. Possible effects on the occurrence of ophthalmia and on the depletion time, with the presence or absence of fat in the basal diet during depletion, have been observed by Nelson and Swanson ('32), Nakahara and Yokoyama ('28), and Culhane ('33); whereas Green ('34) and Lease and Steenbock ('39) observed that

[1] Approved by the director of the Michigan Agricultural Experiment Station for publication as Journal Article No. 465 (N. S.).

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widely varying percentages of fat in the basal diet did not appreciably affect survival time of rats on an A-free diet. These studies related to the depletion of vitamin A stores in the body. In the present investigation, body stores of vitamin A were depleted with the animals receiving a constant level of fat in the diet. The effect of the level of fat intake upon the utilization of graduated levels of vitamin A was then studied by means of diets containing different percentages of fat but adjusted to the same energy or calorie value by dilution with agar-agar. Rate of growth and incidence of "abscessed" areas were the criteria used to measure the response to graduated levels of vitamin A of 264 animals observed during the 2-year period, 1936 to 1938.

EXPERIMENTAL PROCEDURE

Triads of rats of the same sex and litter were started on the vitamin A deficient diet (table 1) at a weight of 50 to 55 gm. and an age of 25 to 28 days. At depletion, as determined by failure to gain in weight for 1 week and evidence of ophthalmia, the basal ration of the triads of rats was changed, each

TABLE 1

Composition of basal rations[1]

INGREDIENTS	DIET USED DURING DEPLETION	DIETS FOR EXPERIMENTAL PERIOD		
		Diet 0	Diet 5	Diet 10
Fat (Refined cottonseed oil): %	5	0	5	10
Casein[2]: %	18	18	18	18
Salt mixture[3]: %	4	4	4	4
Corn starch[4]: %	73	78	67	56
Agar-agar: %	0	0	6	12
Calories per 100 gm., calculated	409	384	385	386
% calories from fat	11.0	0	11.7	23.0

[1] Throughout the entire experiment 0.5 gm. bakers' yeast was fed daily as a supplement to supply the B vitamins.

[2] Crude casein purified for vitamin A assays according to Sherman, H. C., and Smith, S. L. The Vitamins, p. 257, 1931, second edition, Chemical Catalog Company.

[3] Osborne, T. B., and L. B. Mendel, J. Biol. Chem., vol. 32, pp. 309–323, 1917.

[4] Irradiated in a thin layer for 15 min. with a quartz mercury vapor lamp.

member of the triad receiving a different basal diet. The compositions of the three diets containing, respectively, 0, 5, or 10% fat and adjusted to the same caloric value through the addition of agar-agar, are shown in table 1. Thus, for the depletion period, each member of a triad received the same basal ration containing a constant level of fat, whereas during the experimental period, each member of the triad received a different basal diet, the three experimental diets being of the same caloric value per gram but differing in per cent of calories derived from fat. Each member of a triad, however, received the same level of vitamin A supplement, 0, 1, 3, or 6 International Units. Refined cottonseed oil was used throughout the experiment as the source of fat in the basal diet and as the diluent for the U. S. P. reference cod liver oil supplements (U. S. P., 1935). The same methods of preparation of reference cod liver oil dilutions and of administration of supplement employed in the previous work were used in this study. During the 6-week experimental period, the animals were weighed weekly, but the food consumption was checked daily. A quantitative uniformity in food consumption was insured by restriction of the food intake of two members of a triad to the level of the poorest feeder in that triad.

Gross autopsy findings were tabulated at the end of the experiment. As had been found in the previous study, a numerical total of the incidence of accumulations of keratinized epithelial cells, which were called "abscesses" seemed the most reliable and objective estimate of the utilization of the vitamin A.

DISCUSSION OF RESULTS

Male and female triads of rats were depleted on a diet containing 5% of fat in an average of 7.0 weeks. The growth plateau occurred at an averaged weight of 136.5 gm. Food intake averaged 58.6 gm. per week during the depletion period.

Effect upon growth

In table 2 are given summarized data for the 6-week experimental period for both sexes combined for the 2 years. Animals of the negative control group were usually found in a state of death rigor and an accurate measurement of length from tip of nose to tip of tail was not possible. Consequently, too few measurements of length of negative control animals were obtained to warrant presentation here. All of the average results were tested by the "t" test according to the formula "t" $= \frac{m - m_1}{\sqrt{\frac{(\gamma)^2}{n} + \frac{(\gamma_1)^2}{n_1}}}$ and the results are presented in table 3.

It will be noted from table 2 that, in general, with the negative groups, there was a tendency for a lower caloric intake and a greater loss of weight with a higher level of fat in the diet. There was also a shorter survival time on the higher levels of fat, the average length of survival time on the 0, 5, and 10% levels of fat being 16.1, 12.2, and 14.5 days, respectively. However, when these differences in average weight change during survival and number of "abscesses" at autopsy were tested by the "t" test, they were not found to be significantly different on different levels of fat (table 3, part 1). These results, then, regarding the relation of levels of fat in the diet to complete depletion of body stores of vitamin A are in agreement with findings of Green ('34) and Lease and Steenbock ('39).

Reasonably uniform caloric intakes were maintained within groups of animals at all levels of vitamin A administration. At the level of 1 I. U. of vitamin A, the groups of animals receiving 10% fat had an average weekly caloric intake which was 5.47 calories greater than that consumed by rats receiving no fat. However, it was expected that this increase in the calories consumed by rats receiving 10% fat would give only about 0.03 gm. gain in body weight per week, if calculated from the average gain per calorie ingested by these animals (0.005 g $\times$ 5.47). Actually, the 10% fat group showed a 2.5-gm. increase in weight per week over the 0% fat group on this

TABLE 2

Average weights, food consumptions, and autopsy findings on all triads during 6-week experimental period.

YEAR AND DESCRIPTION OF TRIADS	LEVEL OF VITAMIN A	ANIMAL	WEEKLY AVERAGE			GAIN PER CALORIE INGESTED	LENGTH RAT AT END OF EXPER.	"ABSCESSES" EVIDENT AT AUTOPSY [2]
			Food intake [1]	Calorie intake	Weight change [1]			
	units [3]	*no.*	*gm.*	*cal.*	*gm.*	*gm.*	*inches*	
1936–1938 Males and Females								
0% Fat	0	22	26.0 ± 1.54	99.84	—14.9 ± 1.41	—0.149		4.0 ± 0.30
5% Fat	0	22	23.3 ± 1.51	89.71	—15.2 ± 1.42	—0.169		4.1 ± 0.25
10% Fat	0	22	22.1 ± 1.81	84.31	—19.1 ± 1.48	—0.227		4.2 ± 0.29
1936–1938 Males and Females								
0% Fat	1	22	45.5 ± 0.91	176.72	—1.5 ± 0.71	—0.008	13.37 ± 0.15	1.1 ± 0.20
5% Fat	1	22	46.8 ± 0.87	180.18	+0.7 ± 0.72	+0.004	13.45 ± 0.16	1.1 ± 0.29
10% Fat	1	22	47.2 ± 0.76	182.19	+1.0 ± 0.63	+0.005	13.47 ± 0.11	1.1 ± 0.27
1936–1938 Males and Females								
0% Fat	3	22	56.4 ± 1.14	216.58	+3.6 ± 0.71	+0.017	13.68 ± 0.12	1.0 ± 0.29
5% Fat	3	22	57.3 ± 0.97	220.61	+5.2 ± 0.74	+0.024	13.88 ± 0.11	0.5 ± 0.14
10% Fat	3	22	57.2 ± 0.98	220.79	+6.2 ± 0.75	+0.028	13.85 ± 0.11	0.6 ± 0.21
1936–1938 Males and Females								
0% Fat	6	22	63.2 ± 1.21	242.69	+5.5 ± 0.76	+0.023	13.86 ± 0.15	0.6 ± 0.17
5% Fat	6	22	62.6 ± 1.20	241.01	+7.4 ± 0.81	+0.031	13.93 ± 0.14	0.8 ± 0.24
10% Fat	6	22	63.1 ± 1.16	243.57	+8.6 ± 0.75	+0.035	14.18 ± 0.17	0.7 ± 0.19

[1] Arithmetic mean ± standard error.

[2] Areas that most frequently showed "abscesses" were middle ear, base of tongue, lymph glands in neck, salivary glands, and genito-urinary tract.

[3] International Unit.

in the diets used in this study made possible a more complete absorption of vitamin A, which in turn aided in better utilization of the calories ingested, as indicated by the slightly higher gains per calorie on the fat-containing basal rations.

As would be expected, increasing the level of vitamin A resulted in increased gains in weight, irrespective of level of fat in the basal diet, and the more highly significant differences in weight response occur between the wider ranges of vitamin intake. It is recognized that a part of this increased gain in weight with each increase of vitamin intake is due to differences in caloric intake. In a previous study (Muelder and Kelly, '41), it was demonstrated that the percentage of gain in weight from caloric intake increased with each elevation of vitamin intake, the average per cent of the gain in weight due to caloric intake at these respective levels being 59% at the 1-unit level, 64% at the 3-unit level, and 66% at the 6-unit level of vitamin intake.

It has been suggested (Orr and Richards, '34) that gain in weight is not synonymous with growth, and that animals continued to grow in length in spite of insufficient vitamin A in the diet. Measurements of the rats from the tip of the nose to the tip of the tail were made at the end of the experimental period of all animals on 1-, 3-, and 6-unit levels of vitamin intake. The mean lengths for each group are presented in table 2 and results of the "t" tests for significant differences in these lengths are recorded in table 3. In no case was there a significant difference in length due to a difference in percentage of fat in the basal diet, and only at the 10% level of fat was there a highly significant difference in length between the 1- and 6-unit levels of vitamin intake. Differences in length of rat at the different levels of vitamin intake were not as pronounced as the differences in weight gains. The fact that the differences in length of the rat between the 1- and 6-unit group on all three basal diets and also between the 1- and 3-unit groups on the basal diets containing 5 and 10% fat were significant would indicate that level of vitamin intake does affect growth in length.

Effect on incidence of "abscesses"

The summarized data on incidence of accumulations of keratinized epithelial cells are presented in table 2 as the average number of "abscesses" evident at autopsy. The levels of fat in the diets used in this experiment had no effect on protection against the development of areas of cell proliferations. The differences in response on the different basal diets were not statistically significant at any level of vitamin intake (part I, table 3).

As was found in the previous report, the administration of even 1 International Unit of vitamin A to groups of rats produced a highly significant reduction in number of "abscesses" over those observed for groups receiving no vitamin A. The administration of either 3 or 6 units showed a slight reduction in number of "abscesses" below those exhibited by groups of animals given 1 unit of vitamin A; but again these differences are not statistically significant, as shown by the "t" tests.

Although negative control groups demonstrated a slightly higher incidence of "abscesses" in this experiment than in the previous experiment (4.0, 4.1 and 4.2 as compared with 3.2, 3.5 and 3.7), the administration of 1, 3, or 6 International Units of vitamin A was equally as effective in reducing incidence of such areas as similar levels had been previously. Averages of 1.1, 0.7, and 0.7 "abscesses" for groups of animals on the 1-, 3-, and 6-unit levels of vitamin A in this experiment compare favorably with 1.5, 1.1, and 1.1 "abscesses" for groups on the same respective levels of vitamin A in the previous experiment.

SUMMARY

The influence of the level of fat in isocaloric basal diets upon the utilization of vitamin A has been studied by means of rate of growth and incidence of accumulations of keratinized cells or "abscesses" in carefully controlled triads of rats.

Inclusion of 10% of fat in the basal diet aided absorption of vitamin A sufficiently to produce statistically significant gains in weight over a basal diet containing no fat, but not over a

basal diet containing 5% of fat. For the levels of fat and units of vitamin A intake used in this experiment, unitage of vitamin intake was a more important factor in the production of highly significant gains in weight than level of dietary fat.

Growth in length was not affected by level of fat in the basal diet, but a significant increase in length was obtained by increased vitamin A intake.

The level of fat in the basal diet showed no statistically significant influence upon "abscesses," at the levels of fat and vitamin intake used in this experiment. Administration of 1, 3, or 6 International Units of vitamin A to groups of animals produced highly significant reductions in number of "abscesses" as compared to incidence of "abscesses" in groups receiving no vitamin A, although there were no statistically significant differences in number of such areas between these levels of vitamin A.

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THE RESPIRATORY QUOTIENT OF PROTEIN OF THE DALMATIAN DOG [1]

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The metabolism of the Dalmatian dog is characterized by a larger excretion of uric acid than that of other breeds (Benedict, '15–'16, '16; Wells, '18). It has been shown (Henry, Magee and Reid, '34) that if alanine is oxidized to uric acid, as in birds, the theoretical respiratory quotient is 0.667, but if alanine is oxidized until urea is the end product, then the respiratory quotient (R. Q.) is 0.83. These two facts led us to study the total metabolism of a Dalmatian dog with particular reference to the R. Q. and the relation of the excretion of uric acid to the R. Q., as well as the influence of ingestion of protein upon the R. Q.

EXPERIMENTAL

The respiratory exchange of an adult, female Dalmatian dog (aged 7 years and weighing 15 kg.) was measured by an open-circuit respiration chamber, and the outgoing air was analyzed on a Carpenter gas-analysis apparatus. The observations included the effects of several days of fasting and the effects of ingestion of different amounts of casein or raw beef (warmed to body temperature), either immediately before or several hours prior to the experiments. Differences in the time relationships with respect to ingestion of food were purposely

[1] A preliminary report of this study was presented at the 35th Annual Meeting of the American Society of Biological Chemists in Chicago, Illinois, on April 18, 1941. J. Biol. Chem., vol. 140, p. xxiii (1941).

made, to establish whether there was storage of either fat or sugar from protein with a subsequent combustion of the same, which would be indicated by the level of the R. Q. at some period of time following the ingestion of protein. Half-hour periods of measurement, continuing for 1½ to 3 hours, were made on from 12 to 16 days in two series of experiments 11 months apart. In the first series (1937) the dog was in early pregnancy, although this was not known when the study began. Her pregnancy terminated on August 14, 1937, when she gave birth to three puppies. At the start of the 1937 series the urine was collected only in 24-hour periods, but on June 28, 1937, and thereafter and also in 1938 the dog was catheterized immediately preceding and following the respiratory exchange measurements, and the urine was collected for the rest of the day either by catheterization or by voluntary voiding.

DISCUSSION OF RESULTS

The results of the observations on total metabolism, R. Q., and urinary nitrogen elimination are shown in table 1. The non-protein R.Q.'s were calculated in the conventional manner, and the table shows that, with the exception of the beginning of the fasting periods, they are for the most part below 0.71, the R. Q. of fat, and in general the higher the percentage of oxygen used to oxidize protein, the lower is the non-protein R. Q. This is particularly true of the last two values in the table.

The urine did not show any signs of acidosis by a qualitative test, and the percentage of ammonia in relation to the total nitrogen in the urine was not high enough in any case to indicate the presence of acidosis. Therefore it was assumed that the combustion of fat was normal in this dog. Calculations were then made as to what the R. Q. of protein would be if this were the case. For this calculation it was assumed that the factor for oxygen for protein combustion per gram of urinary nitrogen is correct. From the total oxygen consumption was deducted the oxygen used in protein combustion. The remainder was considered to be that for fat combustion.

TABLE 1

Respiratory exchange and urinary nitrogen elimination of a Dalmatian dog.

DATE	CONDITION	EXPERIMENT BEGAN	DURATION	O_2 PER HALF HOUR	URINARY NITROGEN PER HOUR	RESPIRATORY QUOTIENT		PER CENT OF O_2 USED TO OXIDIZE PROTEIN	RECALCULATED PROTEIN R. Q.
						Total	Non-protein		
1937			*hr.*	*l.*	*mg.*				
June 17	24 hrs. without food	10:50 A.M.	2	2.72	...	0.891	....	...	....
June 18	48 hrs. without food	10:00 A.M.	2½	2.72	...	0.714	....	...	....
June 21	48 hrs. without food	10:00 A.M.	2½	2.96	124[1]	0.724	0.713	12.4	....
June 22	3 days without food	10:00 A.M.	2½	3.02	124	0.714	0.701	12.1	0.739
June 23	4 days without food	9:45 A.M.	2½	2.12	131	0.717	0.697	18.3	0.743
June 24	5 days without food	10:00 A.M.	2½	2.23	146	0.696	0.671	19.2	0.653
June 25	6 days without food	9:45 A.M.	2½	2.32	133	0.692	0.670	16.9	0.632
June 26	7 days without food	9:22 A.M.	2½	2.70	133[1]	0.699	0.683	14.5	0.656
June 28	9 days without food	9:37 A.M.	1½	2.39	158	0.703	0.677	19.6	0.677
June 28	25 mins. after 30 gm. casein	12:02 P.M.	3	2.52	249	0.715	0.678	29.1	0.726
June 29	17 hrs. 40 mins. after 70 gm. casein	9:40 A.M.	1½	2.52	237	0.701	0.661	27.8	0.681
June 30	18 hrs. 45 mins. after 100 gm. casein	9:45 A.M.	1½	2.34	416	0.737	0.665	52.5	0.759
July 1	ca. 18 hrs. after 175 gm. casein	10:00 A.M.	1½	2.30	515	0.734	0.598	66.1	0.743
July 2	{ 20 hrs. after 300 gm. beef 16 hrs. after 300 gm. beef	10:00 A.M.	1½	2.41	401	0.726	0.652	49.1	0.740
July 3	39½ hrs. without food	9:35 A.M.	1½	2.38	545	0.749	0.636	67.6	0.765
July 6 July 7	{ ca. 22 hrs. after 400 gm. beef 18 hrs. after 400 gm. beef	10:00 A.M.	1½	2.48	...	0.721	....	...	....
1938	{ ca. 22 hrs. after 400 gm. beef 17 hrs. after 400 gm. beef	10:00 A.M.	1½	2.43	441	0.740	0.668	53.7	0.764
May 3	25½ hrs. without food	9:53 A.M.	2	2.68	204	0.799	0.797	22.5	....
May 4	13½ hrs. without food	9:22 A.M.	2	2.65	215	0.829	0.837	24.0	....
May 5	14¼ hrs. without food	9:14 A.M.	2	2.63	142	0.805	0.805	16.0	....
May 6	38¼ hrs. without food	9:16 A.M.	2	2.49	143	0.704	0.684	16.9	0.682
May 7	62½ hrs. without food	9:29 A.M.	2	2.62	126	0.692	0.674	14.1	0.613
May 8	3 days 14½ hrs. without food	9:26 A.M.	2	2.46	125	0.689	0.669	15.0	0.605
May 9	4 days 14½ hrs. without food	9:25 A.M.	2	2.53	122	0.683	0.663	14.3	0.574
May 10	34 mins. after 350 gm. beef	9:00 A.M.	2½	2.88	305	0.682	0.626	31.3	0.638
May 10	4 hrs. after 350 gm. beef	12:30 P.M.	3	2.82	550	0.720	0.608	57.7	0.727
May 11	25 hrs. after food	9:35 A.M.	2	2.29	134	0.695	0.672	17.2	0.638
May 12	35 mins. after 500 gm. beef	9:14 A.M.	1½	3.25	410	0.713	0.659	37.3	0.717
May 12	3 hrs. 46 mins. after 500 gm. beef	12:25 P.M.	3	3.34	673	0.742	0.650	59.5	0.761
May 13	10 hrs. 49 mins. after 350 gm. beef	9.49 A.M.	2	2.56	468	0.697	0.572	54.0	0.688
May 14	10 hrs. 40 mins. after 500 gm. beef	9:25 A.M.	2	2.72	714	0.720	0.430	77.7	0.721

[1] Assumed.

Multiplication of this remainder by the R. Q. of fat (0.71) gave the carbon dioxide produced in the fat combustion. Subtraction of this from the total carbon-dioxide production gave that ascribable to protein combustion, and the ratio of this latter to the oxygen used in protein combustion gave the recalculated R. Q. of protein. The results are given in the last column of the table and show, in general, much more uniform quotients than are found in the column for the non-protein R. Q.'s.

Calculations of the effect upon the R. Q. of assumed errors in measurement of the respiratory exchange and of the urinary nitrogen did not show that these low non-protein quotients could be due to errors in measurement. The experiments were purposely spaced at varying times after the ingestion of the protein-containing foods, but as no experiment showed at any time quotients high enough to indicate a period of carbohydrate combustion, it is concluded that the low non-protein R. Q.'s cannot be the result of formation of sugar from protein. The excretion of uric acid was not high enough in any case to account for the lowering of the non-protein R. Q. Therefore the hypothesis is proposed that the R. Q. of protein of the Dalmatian dog, although varying with the condition of the animal with respect to fasting and ingestion of food, is lower in general than the usually accepted R. Q. of 0.81 for protein.

SUMMARY

The respiratory exchange of a Dalmatian dog was measured during fasting and after the ingestion of casein or meat in various amounts. The urine was collected by catheterization. The majority of the non-protein R. Q.'s were below 0.71, a fact that cannot be ascribed to formation of sugar from protein or to excretion of uric acid. As the urine showed no signs of acidosis, the protein R. Q.'s were calculated on the assumption that the fat metabolism was normal. They were all well below 0.81, and the hypothesis is advanced that the R. Q. of protein of the Dalmatian dog is lower than that used in general for protein.

The respiratory exchange measurements were made by Mr. Basil James and the urine analyses by Mr. Martin Stankard.

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VITAMIN A REQUIREMENTS IN THE RAT. THE RELATION OF VITAMIN A INTAKE TO GROWTH AND TO CONCENTRATION OF VITAMIN A IN THE BLOOD PLASMA, LIVER AND RETINA

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TWO FIGURES

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In 1939 Goss and Guilbert reported on the minimum vitamin A requirements for the rat. These investigators used as an indicator of vitamin A deficiency the appearance of cornified epithelial cells in smears obtained from the vagina and came to the conclusion that 18 to 22 international units (I. U.) of vitamin A per kilogram of body weight represented the daily minimum requirement. This level of intake not only prevented the occurrence of cornified cells in the vaginal smear, but also brought about fairly good growth and, to all outward appearances, the animal seemed normal in every way. However, at this unitage no vitamin A was detected in the liver, the chief reservoir of vitamin A in the body.

The present study was undertaken to obtain information on the optimal vitamin A requirements for the rat, i.e., that amount of vitamin A intake which will bring about the most favorable nutritional state. For the appraisal of the vitamin A status, we used the following criteria: (1) growth; (2) vitamin A blood level; (3) liver reserve; (4) vitamin A concentration in the retina. A previous report on the relation of vitamin A intake to blood level and to liver storage has appeared elsewhere (Lewis, Bodansky, Falk and McGuire, '41).

EXPERIMENTAL

One hundred and sixty-four albino rats, 3 to 4 weeks of age, obtained from the same colony and having been reared under identical conditions, were divided into groups and were fed the following supplements of vitamin A daily: 0, 1, 2, 10, 25, 50, 100 and 1000 I. U., respectively. The basal diet was devoid of vitamin A and had the following percentage composition: casein 18, salt mixture (Osborne and Mendel) 4, irradiated dry yeast 8, starch 65, hydrogenated cottonseed oil 5. The vitamin A supplement used for rats receiving 100 units or less daily was a reference cod liver oil obtained from the U. S. Pharmacopoeia Committee, whereas the animals receiving 1000 units daily were given a special preparation of vitamin A which was concentrated by the Hickman process ('37)[1].

The rats were weighed twice weekly and after they had received these vitamin A supplements for 6 weeks, they were sacrificed by exsanguination 24 hours after the last dose of the vitamin. The vitamin A contents of their bloods, livers and retinas were then determined.

Depletion experiments, which will be described later in the paper, were carried out on sixty-one additional rats.

Determination of vitamin A in the blood. The method employed was based on the Carr-Price reaction and, except for slight modification, was in accordance with the technique described by Kimble ('39). As the diet was devoid of carotene, this substance was not found in the blood, liver or retina. It was necessary to pool the blood of two or three rats in order to have a sufficient quantity of blood for a vitamin A determination.

Determination of vitamin A in the liver. To a sample of liver[2] weighing between 0.5 to 1 gm., 0.3 to 0.5 ml. of 60% KOH and 5 ml. of 95% alcohol were added and the mixture

[1] The vitamin A concentrate was obtained through the courtesy of the Winthrop Chemical Company and contained 200,000 international units per gram.

[2] At the lower vitamin A intakes the whole liver, or a considerable portion thereof, was used for analysis.

was placed in a water bath at 70° C. until the tissue disintegrated, which usually occurred within 15 to 20 minutes. The remainder of the procedure was the same as that employed in the determination of the vitamin A content of the blood.

Determination of vitamin A in the retina. The eyes were removed in bright daylight[3] immediately after the rats were sacrificed. A vertical section through the eyeball was made just distal to the lens, the anterior portion of the eye being discarded and the posterior portion being placed in a 4% alum solution for 5 minutes[4]. The retinas were then dissected from the remaining tissues and were placed in a previously weighed centrifuge tube containing 5–10 ml. of distilled water. The tube was shaken vigorously to free the retinas from any alum, and then centrifuged. The supernatant liquid was decanted after centrifugation and this procedure was repeated three times. After the last centrifugation, water adhering to the sides and the bottom of the tube, as well as to the packed retinas, was removed with small pledgets of filter paper until the weight of the retinas became constant. The retinas of at least six rats were required to carry out a vitamin A determination; the average weight of the retinas of six rats varied from 60 to 110 mg. depending upon the size of the rats.

Five milliliters of 95% alcohol and 0.1 ml. of 60% KOH solution were then added to the retinas and the mixture was placed in a water bath at 50–70° C. for 10 to 15 minutes, at the end of which time complete disintegration of the retinas usually resulted. The remainder of the procedure was the same as that used for the determination of vitamin A in the blood.

[3] The vitamin A content of the retina was found to be in the same range whether the eyes were removed in the dark from fully dark adapted animals or whether they were removed in bright daylight from rats who had been previously exposed to a bright light (100-watt lamp) for 5 minutes.

[4] The question may be raised whether alum causes any destruction of vitamin A in the retina. Experiments which we carried out revealed that the treatment of the retina with 4% alum causes no destruction of vitamin A and does not interfere with the determination of vitamin A in the procedure we employed.

RESULTS

The relation of vitamin A intake to growth. The rate of growth of the various groups of rats is shown in figure 1. It will be observed that the group receiving no vitamin A gained fairly well for the first 2 weeks, but after this period there was a slowing up in the rate of gain and from the fourth to the sixth week the weight remained stationary. Many of the rats in this group showed evidences of xerophthalmia after having received the basal diet for 6 weeks.

The rate of gain in weight among groups of rats receiving 1, 2, 10 and 25 units, respectively, was directly related to the

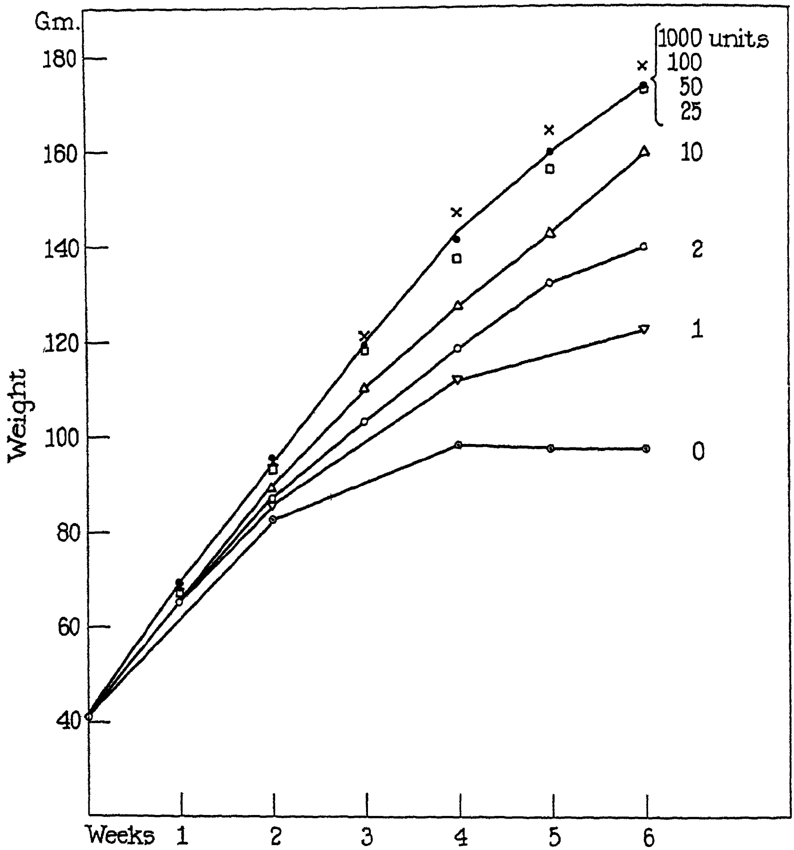

Fig. 1 The relation of vitamin A intake to growth.

intake. Thus, each increase in the amount of vitamin A fed resulted in a corresponding increase in the rate of gain in weight. On the other hand, increasing the intake above 25 units to levels of 50, 100 and 1000 units daily did not bring about a significant improvement in the weight curve.

Relation of vitamin A intake to blood level and to liver storage. The relation of vitamin A intake to vitamin A blood level and to liver storage is shown in table 1. When the intake was less than 50 units daily, the vitamin A concentration of

TABLE 1

Relation of vitamin A intake in the rat to vitamin A blood level, liver storage and vitamin A concentration in the retina.

VITAMIN A GIVEN DAILY FOR 6 WEEKS	ANIMALS IN GROUP	BODY WEIGHT		VITAMIN A		
		Initial (average)	At end of six weeks (average)	In blood plasma (average)	In liver (average)	In retina [1] (average)
I. U.		*gm.*	*gm.*	*I. U./100 ml.*	*I. U./gm.*	*I. U./gm.*
0	13	45	98	0	0	14
1	11	44	119	7	0	—
2	31	40	140	14	0	20
10	31	40	159	35	0	25
25	16	41	172	69	3	20
50	15	41	172	100	34	—
100	21	42	173	112	113	26
1000	26	43	177	110	1270	25

[1] The average concentration of vitamin A in the retina for the group receiving no supplement of vitamin A was computed from analyses carried out in forty-two rats, and for the groups receiving 2 units daily, retinal determinations were done in ninety rats.

the blood was directly related to the vitamin A intake. Increasing the feeding above 50 units daily had no appreciable effect on the vitamin A level in the blood, the average value being 112 units for rats receiving 100 units daily and 110 units for those receiving 1000 units daily. Thus, the minimum intake which brought about maximal blood levels was 50 units daily.

Storage of vitamin A in the liver was not found in any of the rats receiving 10 units or less daily. A small amount of vitamin A was detected in the livers of those receiving the 25 unit intake, an average of only 3 units per gram of liver

having been found. Increasing the intake above 25 units resulted in corresponding increases in the vitamin A storage of the liver. Thus, in contrast to the vitamin A content of the blood where maximal values were reached with an intake of 50 units, the liver concentration of vitamin A continued to mount as the daily intake was increased from 25 to 1000 units.

Relation of vitamin A intake to concentration in the retina. The vitamin A concentration in the retina is of particular interest because, as shown by Wald ('35), this vitamin plays an important role in the photochemical processes which take place in this organ during dark adaptation.

Vitamin A determinations of the retina were carried out on groups of rats receiving daily 0, 2, 10, 25, 100 and 1000 units respectively. The results of these analyses (table 1) revealed approximately the same values for rats receiving 2 or more units daily. On the other hand, rats receiving no vitamin A in their diets for a period of 6 weeks showed a diminished amount of vitamin A in their retinas.

It is of interest to analyze the retinal concentrations obtained within each group. Of seven determinations carried out in rats receiving no vitamin A supplements, five values were 15 units or lower. On the other hand, in the group of rats receiving 2 units daily, four of fifteen analyses revealed values below 15 units, and in the groups receiving 10, 25, 100 or 1000 units, one of fourteen analyses was below 15 units. Thus, in the majority of instances the 2-unit intake brought about maximal vitamin A concentrations in the retina.

Effect of enormous doses on vitamin A concentration in the blood, retina and liver. In view of the fact that the daily ingestion of as many as 1000 units of vitamin A did not bring about higher blood or retinal concentrations than did the feeding of considerably smaller amounts, the question arose as to whether it is possible to bring about abnormally high concentrations in the blood and retina by feeding enormous amounts of vitamin A. We therefore gave twenty rats 100,000 units of vitamin A daily for 4 weeks. The animals were sacrificed 48 hours after the last dose in order to be sure that the

blood concentration was at a post-absorptive level. Vitamin A determinations were then carried out on the bloods, retinas and livers. The results of this experiment revealed abnormally high concentrations of vitamin A in the blood and in the retina, the average blood level being 478 units per 100 cc. of plasma, and the average retinal content being 75 units per gram. The amount of vitamin A found in the liver was enormous, the average value being 19,200 units per gram. It should be mentioned that after receiving this high vitamin A intake for a few days, the animals began to lose weight, and in the course of 2 to 3 weeks became emaciated and lethargic, and several of the rats suffered a loss of hair; six died before the completion of the experiment.

The effect of vitamin A depletion on the concentration of vitamin A in the blood, retina and liver. In order to ascertain the relation of the amount of vitamin A stored in the liver to the maintenance of high blood and retinal levels during periods of depletion, three groups of rats were fed varying quantities of vitamin A and were then placed on a diet devoid of this vitamin. The first group, consisting of eighteen rats, was fed 100 units of vitamin A daily for 2 weeks; the second group, consisting of twenty-four animals, was given 100 units daily for 6 weeks and the third group of nineteen animals was fed 1000 units daily for 6 weeks. Prior to depletion, six animals from each group were sacrificed and the vitamin A content of their bloods, livers and retinas was determined.

After the vitamin A-free diet had been given for periods of 6 and of 9 weeks, six rats from each group were sacrificed and the vitamin A content of the livers, bloods and retinas was then determined. In addition, six animals in the second group were kept on a vitamin A-free diet for 20 weeks, at the end of which time similar analyses for vitamin A were carried out.

The results of this experiment are given in figure 2. It will be noted that, whereas there was a considerable drop in the blood levels in the first two groups of rats, in the third group,

which had an exceedingly large reserve of vitamin A in the liver, the blood level continued to remain high despite the administration of a vitamin A-free diet for 9 weeks. Apparently, the large stores of vitamin A in the livers of the third group maintained the bloods at optimal levels.

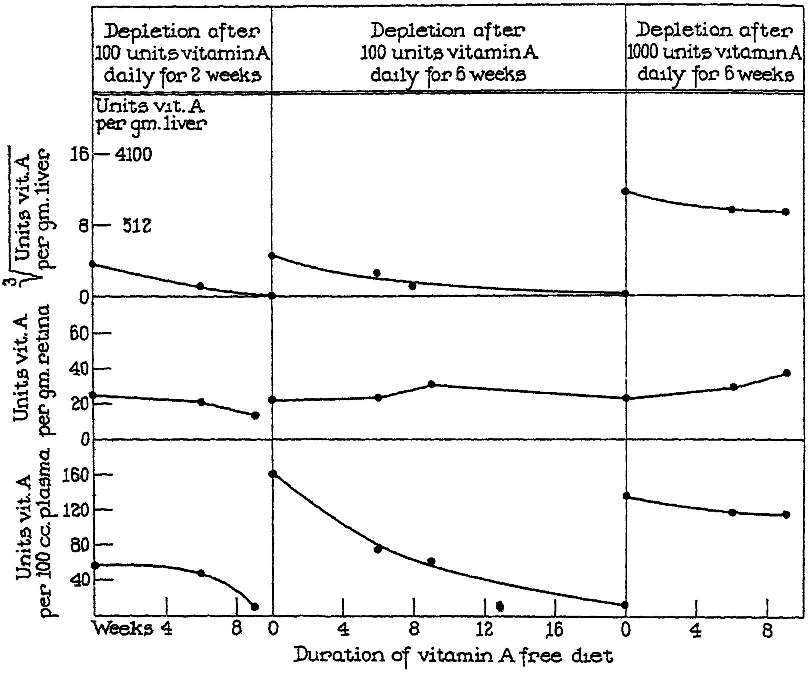

Fig. 2 The effect of a vitamin A-free diet on the concentration of vitamin A in the blood, in the liver and in the retina of rats which were previously fed varying amounts of vitamin A.

The retinal concentrations of vitamin A remained high in all three groups of rats throughout the greater part of the depletion period. In the first group of rats, which had a small storage of vitamin A at the beginning of the depletion period, the retinal concentration was observed to be low, 14 units per gram, only after the vitamin A-free diet had been given for 9 weeks, at which time most of the animals showed a loss of weight and were suffering from xerophthalmia. On the other

hand, in the second group of rats, which had a moderate reserve at the beginning of the depletion period, the retinal concentration remained high even after 20 weeks on a vitamin A-free diet, at which time the blood level was low and the liver was devoid of vitamin A. In this connection it should be added that Popper ('40) also found that during periods of depletion the retina contained vitamin A long after other tissues had been depleted. In Popper's investigation the organs were examined microscopically under ultra-violet light and the amount of vitamin A was estimated by the degree of green fluorescence.

DISCUSSION

Under the conditions of this experiment daily feedings of 2 units were required to prevent gross and histological evidences of vitamin A deficiency[5] and this intake may be regarded as the minimum vitamin A requirement. At this level of feeding, however, the blood concentration of vitamin A was markedly reduced and the liver contained no vitamin A reserve. As the 2-unit group received about 20 units of vitamin A per kilogram of body weight, the minimum requirement was the same as that previously reported by Goss and Guilbert ('39).

As previously stated, most of the rats whose daily intake was 2 units had maximal vitamin A concentrations in the retina. Although we did not carry out dark adaptation tests, it is probable that the animals in this group would have had normal vision in the dark. In this connection, it is of interest to mention that an intake of approximately 20 units per kilogram of body weight was found just sufficient to permit normal dark adaptation in cattle, swine and sheep (Guilbert, Miller and Hughes, '37), and in infants (Lewis and Haig, '39).

The amount of vitamin A which was required to bring about optimal growth in the rat was considerably higher than the

[5] We are indebted to Dr. Henry Brody of the Department of Pathology of Beth Israel Hospital for the histological examinations.

minimum requirement. For optimal gain in weight, a daily intake of 25 units was necessary. Since we did not employ levels of intake between 10 and 25 units daily, it is possible that amounts lower than 25, but greater than 10 units would also have resulted in optimal growth. Thus, the optimal intake, as judged by gain in weight, lies between five to twelve and one-half times the minimum requirement.

It may be recalled that the maximal blood concentrations averaged about 100 units per 100 ml. of blood plasma and were attained when the amount of vitamin A ingested daily was 50 units. Since we did not study the effect of levels of feeding between 25 and 50 units daily, it is possible that an intake of less than 50, but greater than 25, would also have yielded maximal blood concentrations. Thus, the smallest amount of intake required to bring about maximal levels of vitamin A in the blood is somewhere between twelve and one-half and twenty-five times the minimum requirement.

The question arises whether maximal blood values are also optimal, i.e., the most desirable, physiologically. The fact that, in the depletion experiments, rats having large quantities of vitamin A in their livers continued to maintain levels of about 100 units of vitamin A per 100 ml. of blood plasma for long periods of time when they were placed on a vitamin A-free diet, would indicate that the organism strives to maintain this level at the expense of the vitamin A reserve in the liver. It would therefore seem that concentrations of about 100 units per 100 ml. represent optimal blood levels.

It is difficult to determine what amount of vitamin A intake is required to bring about optimal concentrations in the liver inasmuch as we have no accurate way of determining what constitutes an optimal reserve. Certainly an intake of 25 units was too low, as storage in the liver was negligible at this level of feeding. An intake of 50 units daily provided a liver reserve (an average of 34 units per gram) which was of a magnitude that maintained the animal in a good state of nutrition for a period of 6 weeks of depletion, but at the end of 9 weeks, loss in weight and xerophthalmia developed. On the other hand,

rats receiving 100 units daily had a storage of vitamin A in their livers (an average of 113 units per gram) which was sufficient to keep them in a good nutritional state for a period of 20 weeks, during which time a vitamin A-free diet was given. Although we cannot state categorically what intake, under the conditions of our experiment, was optimal insofar as liver reserve is concerned, yet it would seem that an intake of 100 units daily (fifty times the minimum requirement) brought about a very satisfactory store of vitamin A in the liver.

The observations reported in these experiments are of special interest when compared with those noted in infants. Thus, Lewis and Haig ('39) found that the minimum vitamin A requirement in infants, as judged by dark adaptation, was of the same order as that obtained in rats, namely, 20 units per kilogram of body weight. At this intake the infant grew fairly well and appeared normal in every way. However, we have subsequently determined that at this level of intake the concentration of vitamin A in the blood is abnormally low (Bodansky, Lewis and Haig, '41). Optimal blood concentrations occurred in infants as in young rats, when the vitamin A intake was approximately twenty-five times the minimum requirement.

The maintenance of maximal concentrations of vitamin A in the retinas of the rats even when the vitamin A intake was so low as to bring about suboptimal growth, lowering of the vitamin A blood level and depletion of the vitamin A reserve in the liver is an extremely interesting finding. This observation would suggest that the ability of the eye to adapt to darkness (which is presumably dependent upon the vitamin A supply in the retina) is not affected early in the course of vitamin A deficiency. This view is contrary to general opinion, but recent investigations by Lewis, Bodansky and Haig ('41) lend support to this contention. For example, it was observed that several infants who had received a diet almost devoid of vitamin A for several weeks showed low blood concentrations of vitamin A, but dark adaptation remained

normal. Furthermore, twenty-two older children were found to have subnormal levels of vitamin A in the blood associated with normal dark adaptation. In view of these experiences we have come to the conclusion that the blood concentration is a more sensitive indicator of vitamin A deficiency than is the dark adaptation test.

SUMMARY

When vitamin A was fed daily at different levels to groups of 3 to 4 week old rats for a period of 6 weeks, the rate of growth was found to increase until it reached an optimum at an intake of 25 I. U. daily; it remained at this optimum for intakes up to 1000 units daily. The average plasma vitamin A concentration also increased with increasing levels of vitamin A intake, reaching an optimum at an intake of 50 units daily, and remained at this optimum for intakes up to 1000 units daily. There was no liver storage at intakes of 10 units or less daily, slight storage at 25 units, and increasingly larger storage at higher intakes. Retinal concentrations of vitamin A were usually low at zero units intake and reached an optimal value at an intake of 2 units daily. When rats were placed on a vitamin A-free diet, the plasma vitamin A concentration remained high in the animals with large liver stores, but fell rapidly in those with low reserves. The retinal concentration of vitamin A, however, remained high despite the low vitamin A plasma concentration and the absence of vitamin A in the liver. The minimum vitamin A requirement in the rat was 2 units daily or 20 units per kilogram. Optimal growth occurred at 25 units daily intake, optimal blood concentrations at 50 units daily, and good liver reserves at 100 units daily. Minimum and optimum vitamin A requirements are of the same order in the rat and infant.

We wish to express our appreciation to Mr. Bertrand Flusser, to Miss Mildred Weinstock and to Dr. Charles Haig for their cooperation in this study.

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THE EFFECTS OF THE SUBSTITUTION OF BICARBONATE FOR CHLORIDE IN THE DIET OF RATS ON GROWTH, ENERGY AND PROTEIN METABOLISM [1]

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This experiment was designed primarily to study the effects of a deficiency of dietary chloride in relation to the utilization of protein and energy, and serves incidentally to exhibit the effects of prolonged intake of bicarbonate as a prominent dietary constituent.

The published studies of the physiological functions of the chloride ion have been concerned, in the main, with its specific function in gastric juice, and with its role in maintaining the constancy of the internal environment of the tissue cells, with reference both to physiological neutrality and to the distribution of water and electrolytes. Chloride ions are considered as relatively passive participants in the maintenance of the ionic balance of the tissues (Irving and Manery, '36), especially because they do not enter into oxidation-reduction systems, do not form natural organic compounds, and have little specific influence on tissues.

In few investigations, therefore, has attention been given to the effects of a deficiency of dietary chloride on growth, on the utilization of protein, and on its relation to the utilization of dietary energy.

Osborne and Mendel ('18) and St. John ('28) observed that diets containing as little as 0.035 or 0.05% chlorine were

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quite adequate for growth. However, retardation of growth has been noted with more extreme limitations of dietary chloride by Orent-Keiles, Robinson and McCollum ('37) and Marquis ('38). Improvement in growth by the addition of sodium chloride to natural diets has been attributable to the sodium ion more than to the chloride ion (Mitchell and Carman '26; Miller, '26; St. John, '28).

The synthetic chloride-deficient ration used in the present investigation contained 0.02% chloride, and the control ration 0.28% chloride. The chloride in the salt mixture of the latter ration was replaced by equivalent bicarbonate in making up the chloride-deficient ration which, consequently, contained 0.49% bicarbonate. In this quantity, bicarbonate seems to be harmless (Olson and St. John, '25; Kahlenberg, Black and Forbes, '37) and the results obtained with the low-chloride diet are considered to represent mainly the effects of the chloride deficiency.

EXPERIMENTAL

A comparison was made of a chloride deficient diet with one of normal chloride content by means of a metabolism and body analysis experiment using as subjects twelve litter-mate pairs (seven males, five females) of albino rats from a colony originating in the Wistar strain.

The basic experimental diet was of the following percentage composition: sucrose 65, casein 20, hydrogenated vegetable oil 10 [2], cellu flour 2, and mineral salt mixture 3. The salt mixture was basically that of Hubbell, Mendel and Wakeman ('37), the sodium and potassium chlorides of this mixture being replaced by equivalent quantities of bicarbonates of the same cation in the chloride deficient ration.

In addition, each 100 gm. of the ration contained the following vitamin supplements and accessory food factors: carotene 3 mg., viosterol [3] 100 mg. (333 units of vitamin D), thiamine

[2] Crisco.

[3] Squibb, irradiated ergosterol in oil 250 D.

hydrochloride 0.5 mg., riboflavin 0.5 mg., pyridoxine 0.5 mg., calcium pantothenate [4] 0.25 mg. and lecithin [5] 0.15 gm.

By analysis the ration contained 98.6% dry matter, 2.89% nitrogen and 4.66 Calories per gram.

Both rats of a pair received the same amount of the equicaloric diets, the quantity fed being determined daily for each pair by that eaten on the preceding day by the individual which consumed the lesser amount.

The details of experimental technic were essentially as described by Swift, Kahlenberg, Voris and Forbes ('34).

The rats were allowed distilled water ad libitum and the water consumption was determined by weighing the water bottles at appropriate intervals.

Urine and feces were collected daily, and at the end of 10 weeks the paired rats were killed and the bodies analyzed for water, nitrogen, fat and energy.

The total 10-week collections of urine and feces were analyzed for nitrogen and energy. The nitrogen aliquot of the urine was preserved with sulfuric acid and the energy aliquot was dried without preservation. The energy of the dried sample was corrected for the loss of nitrogen in drying on the assumption that it was lost as urea.

Twelve rats (six male, six female) corresponding in age, weight and heritage to the paired rats, were killed and analyzed for alimentary fill, water, nitrogen, fat and energy to represent the body composition of the experimental rats at the start of the experiment. Average analyses for males and females were used separately.

Also, ten rats (seven male, three female) were fed the normal chloride ration ad libitum in order to determine its adequacy for growth.

For the sake of brevity, in discussing the results, the rats which received the chloride-deficient experimental diet are designated the bicarbonate rats, and those which received the diet of normal chloride content are referred to as the chloride rats.

[4] Donated by Merck and Co.

[5] Pfansteil Chemical Co. Pure lecithin.

RESULTS

The rats partook well of the experimental rations and there was no overt evidence of vitamin deficiency.

The rats fed ad libitum on the normal chloride diet thrived and appeared healthy throughout the experiment. The male rats in this group made an average weekly gain of 15.9 gm. on an average food intake of 72.2 gm. per week for the 10 weeks. The female rats made an average gain of 12.6 gm. per week with a food intake of 64.3 gm.

In the experimental group the food intake of the paired rats was invariably limited by the bicarbonate rats. The food intake for the 10 weeks was 531 gm. for the male rat pairs and 508 gm. for the females (table 1). Since the bicarbonate rats

TABLE 1

The effects of the substitution of bicarbonate for chloride in the diet on the growth of rats and utilization of nitrogen over a period of 10 weeks.

	MALES		FEMALES	
	HCO_3^-	Cl^-	HCO_3^-	Cl^-
Feed intake, grams	531.0	531.0	508.0	508.0
Water intake, grams	864.0	705.0	1045.0	699.0
Final body weight [1], grams	147.6	176.7	129.1	158.5
Initial body weight [1], grams	63.8	63.7	53.2	54.0
Total body weight gained, grams	83.8	113.0	75.9	104.5
Fat gained, grams	7.0	12.3	9.5	15.4
Per cent of total gain	8.4	10.9	12.5	14.7
Fat-free tissue gained, grams	76.8	100.7	66.4	89.1
Water gained, grams	51.8	69.9	45.0	61.8
Per cent of fat-free tissue	67.4	69.4	67.8	69.4
Protein gained, grams	19.5	25.0	16.4	21.0
Per cent of fat-free tissue	25.4	24.8	24.7	23.6
Residual tissue [2] gained, grams	5.5	5.8	5.0	6.3
Per cent of fat-free tissue	10.6	5.8	7.5	7.1
Nitrogen intake, grams	15.36	15.36	14.68	14.70
Fecal nitrogen, grams	0.81	0.87	0.80	0.82
Nitrogen digested, grams	14.55	14.49	13.88	13.88
Per cent of intake	94.7	94.3	94.6	94.4
Urinary nitrogen, grams	10.76	9.99	10.73	9.98
Nitrogen balance, grams	3.79	4.50	3.15	3.90
Per cent of intake	24.6	29.3	21.4	26.5

[1] Does not include contents of alimentary tract.

[2] Residual tissue = non-protein, non-fatty, dry substance.

were fed essentially ad libitum, the food intake of the pair-fed chloride rats, compared with those fed ad libitum, was reduced by 26% for the males and 21% for the females.

The water consumption (table 1) was significantly greater for the bicarbonate rats than for the chloride rats. The difference was more marked for the females than for the males, but no explanation is offered for this observation. In one pair of male rats the water consumption was greater for the chloride rat while in two other pairs the amount of water drunk by the chloride and bicarbonate rats was practically the same. There were no such exceptions among the female pairs.

The growth differences and other data were remarkably consistent and, consequently, the average values reported in the tables are quite representative.

Growth

The growth data are presented in table 1. The total weight gained by the bicarbonate rats was, as an average, 73.4% of the total weight gained by their pair-mates on the normal chloride diet. From the analysis of the gains, the bicarbonate rats gained 73.5% as much water, 78% as much protein, 60% as much fat and 87% as much residual (mainly mineral) substance as the chloride rats.

On the basis of the total gain, there was no evident difference in the percentage of gain as water by the bicarbonate and chloride rats, but on the basis of the fat-free gain the percentage of the gain as water by the chloride rats (69.4 ± 0.5 [6]) was significantly greater than the percentage gain as water by the bicarbonate rats (67.1 ± 0.6), and the individual data were characterized by a high degree of consistency.

The percentage gain of protein was significantly greater for the bicarbonate rats than for the chloride rats on the basis of either total weight or the fat-free tissue gained. On the latter basis, protein represented 25.1% of the fat-free tissue gained by the bicarbonate rats and 24.2% of that gained by

[6] Standard deviation.

the chloride rats. Statistically, the odds were 370 to 1 against this difference occurring by chance alone.

The relationship of water and protein has a definite significance with respect to tissue cell structure. From the data of Forbes, Voris, Bratzler and Wainio ('38) the same ratio of water to protein gained prevailed for diets containing 25, 30, 35 or 45% protein. This value was 2.93 gm. of water gained for each gram of protein gained. In the present investigation the ratio of water gained to protein gained was 2.87 ± 0.11 [7] for the chloride rats and 2.70 ± 0.08 for the bicarbonate rats. Statistically the odds are greater than 10,000 to 1 that this difference is significant. Thus, there is no doubt that the tissue cells of the bicarbonate rats were water-poor because of the insufficiency of chloride in the diet.

Fat gained by the chloride rats represented a significantly larger proportion of the total gain than the fat gained by the bicarbonate rats and, conversely, the chloride rats gained a smaller proportion of residual substance.

Utilization of protein

The effects of dietary chloride deficiency on the disposal of dietary nitrogen are presented in table 1. The fecal nitrogen of the bicarbonate rats was significantly lower than that of the chloride rats, but this difference when related to the much greater nitrogen intake appeared to be negligibly small. The difference in the digestibility of nitrogen, therefore, cannot be emphasized.

The urinary nitrogen excretion was about 8% greater in the bicarbonate rats than in the chloride rats and, consequently, the latter showed a significantly higher nitrogen retention than the former. The nitrogen retention for the bicarbonate rats was 82.6% of that for the chloride rats. The corresponding value determined from the body analysis for nitrogen gained was 77.7%. These balances differ slightly in significance since the value determined by the collection of the excreta involves a small error on account of the contamina-

[7] Standard deviation.

tion of the feces with shed hair and scurf, while the balance determined by body analysis involves the error of failure to account for the nitrogen of the shed hair and scurf as having been utilized.

Protein gained (table 1) was computed from the body analysis data for nitrogen gained by using the factor 6.0 for converting nitrogen to protein. The significance of the differences in protein storage has been discussed in connection with the growth data.

Utilization of energy

The results of chloride deficiency as affecting the utilization of energy are presented in table 2. Corresponding to the slightly greater digestibility of nitrogen, the bicarbonate rats, likewise, digested a slightly greater percentage of their energy intake than did the chloride rats. In relation to metabolizable

TABLE 2

The effects of the substitution of bicarbonate for chloride in the diet of rats on the metabolism of energy over a period of 10 weeks.

	MALES		FEMALES	
	HCO_3^-	Cl^-	HCO_3^-	Cl^-
Energy intake, Calories	2477	2477	2368	2371
Fecal energy, Calories	138	168	139	144
Energy digested, Calories	2339	2309	2229	2227
Per cent of intake	94.4	93.2	94.1	93.9
Urinary energy, Calories	76	68	73	67
Non-metabolizable energy of body protein, Calories[1]	24	31	20	26
Metabolizable energy, Calories	2239	2210	2136	2134
Per cent of intake	90.4	89.2	90.2	90.0
Energy of body gain, Calories	180	259	186	269
Per cent of metabolizable energy	8.0	11.7	8.7	12.6
Energy gain as fat, Calories	66	116	90	146
Per cent of total gain	36.7	44.8	48.4	54.3
Energy gain as protein, Calories	114	143	96	123
Per cent of total gain	63.3	55.2	51.6	45.7
Heat production, Calories	2083	1982	1970	1891
Per cent of metabolizable energy	93.0	89.7	92.2	88.6

[1] Grams of nitrogen gained (body analysis) $\times$ 7.45 = Calories of non-metabolizable energy of body protein gained.

energy this difference was compensated, in part, by the higher urinary energy of the bicarbonate rats, but when the non-metabolizable fraction of the body protein stored was considered, the metabolizable energy was 90.2% of the energy intake compared with 89.6% for the chloride rats. Statistically, the odds are 200 to 1 against the difference occurring by chance alone.

However, the chloride rats stored 12.2% of their metabolizable energy as body gain, compared with 8.4% gained by the bicarbonate rats. Conversely, the chloride rats lost 89.2% of their metabolizable energy as heat, whereas the bicarbonate rats lost 92.6% as heat. Thus, the difference in heat production is accounted for by the inability of the bicarbonate rats to retain as large a proportion of their metabolizable energy as did the chloride rats.

The chloride rats exceeded the bicarbonate rats in energy gained both as protein and as fat, in absolute quantities. The percentage of the total energy gain as protein was smaller for the chloride rats than for the bicarbonate rats and, conversely, the percentage of the total energy gain as fat was larger.

DISCUSSION

In summarizing the comparison between the bicarbonate and chloride rats, the following effects may be enumerated as the result of a deficiency of dietary chloride: (1) depression of appetite, (2) increased consumption of water, (3) retarded growth, (4) smaller proportionate gain of fat and water, (5) larger proportionate gain of protein and residual (mainly inorganic) substance, (6) slightly decreased fecal nitrogen, increased urinary nitrogen and decreased nitrogen retention, (7) slightly greater metabolizability of energy, but a lesser gain of body energy and a higher heat production.

Thus, the nutritional effects of a deficiency of dietary chloride are, in many respects, quite similar to the effects of deficiencies of sodium and potassium (Orent-Keiles, Robinson and McCollum, '37; Kahlenberg, Black and Forbes, '37;

Orent-Keiles and McCollum, '41). In fact, the observed effects of chloride deficiency are not materially different from those of a general mineral deficiency, as described by Brooke and Smith ('33), Eppright and Smith ('37), and Kriss and Smith ('37, '38 a, '38 b). The workers last mentioned found that both the basal and the total energy metabolism were considerably greater with rats subsisting on mineral deficient diets than with rats receiving normal control diets. The increased total metabolism was accounted for largely by the increased basal metabolism. The mineral deficient rats oxidized relatively larger proportions of fat and smaller proportions of carbohydrate, while no difference was noted in the protein metabolized.

SUMMARY

In a paired-feeding experiment comparative data on water consumption, growth, energy and protein metabolism, and body composition were secured with rats on a synthetic diet with normal chloride content (0.28%) and rats on the same diet in which the chlorides of the salt mixture were replaced by equivalent quantities of the corresponding bicarbonates. The chloride deficient ration contained 0.02% chloride and 0.49% bicarbonate. At this level of intake, the bicarbonate was considered to be innocuous and the results obtained were attributed to the deficiency of dietary chloride.

In comparison with the rats receiving the normal chloride ration, the chloride deficient rats showed depression of appetite, increased consumption of water, increased heat production and diminished body gain of nitrogen and energy.

There was a smaller percentage gain of water on the basis of fat-free tissue, and the ratio of water gained to protein gained was significantly lower with the chloride deficient rats than with the normal controls.

The prevailing deficiency of chloride ion did not affect the digestion and absorption of nutrient energy but did prominently affect its disposal within the bodies of the rats.

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THE INTERRELATION OF CALCIUM AND FAT UTILIZATION IN THE GROWING ALBINO RAT [1]

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Several recent publications have indicated that the addition of fat to a fat-free diet has a favorable influence on calcium utilization, but variations in experimental conditions and dietary regimes have led to differences in conclusions.

Among the first to investigate this relationship were Holt, Courtney and Fales ('20), who attributed certain instances of poor calcium retention to the formation of insoluble calcium soaps from excess fatty acid in the intestine. Zucker and Barnett ('23) suggested that part of the antirachitic property of natural fats was due to the union of calcium with the fatty acids to form soaps, thereby conserving phosphorus which would otherwise have been excreted as insoluble calcium phosphate. Telfer ('26) noted that the faulty utilization of bone-forming elements in rickets seemed to be due to defective absorption. Westerlund ('34 a, '34 b, '40) fed the pure fats tripalmitin, triolein, tributyrin, and tristearin to adult rats. He noted a deleterious effect of tristearin and tripalmitin on calcium absorption, which was exerted directly in proportion to the molecular weights.

Knudson and Floody ('40) and Jones ('40) found that moderate levels of fat in the diet favored the utilization of calcium.

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The purpose of the present experiment is to obtain further information concerning the physiological relationship between fat and calcium in the rapidly growing animal.

EXPERIMENTAL PROCEDURE

The experimental subjects were twenty-eight normal male albino rats of The Wistar Institute strain. They were selected as quadruplets, four from a litter, 3 days after weaning, at which time the animals were 26 days old and weighed from 48 to 56 gm. Six sets of four litter mates each were selected initially, but due to the abrupt death of one individual, an additional set of four litter mates was chosen in which the rats were 29 days old and averaged 71 gm. in weight. Each rat of a set of litter mates was fed one of the four experimental rations.

The animals were on experiment for a period of approximately 6 weeks, which included a 4-day preliminary period and a 40-day period of excreta collection. The rats were kept in individual metabolism cages designed by Swift et al. ('34) which permitted the separate collection of the feces and the urine. Distilled water was available to the rats at all times.

At the end of the collection period the rats were sacrificed, the gastrointestinal tracts removed, and the acidity of the contents determined with the glass electrode Beckman pH meter.

Calcium was determined by the volumetric method of the Association of Official Agricultural Chemists ('40) in which the calcium is precipitated as the oxalate and titrated with permanganate. Ether extract was determined by the official, direct method of the Association of Official Agricultural Chemists ('40).

Preparation of rations

Four synthetic rations (table 1) were prepared from purified foodstuffs in such manner as to provide, when fed in equicaloric amounts, essentially the same contents of protein, salt

mixture, calcium, phosphorus, and vitamin supplements. The rations, however, contained 5, 15, 28 and 45% fat, respectively. Salts were provided in the Osborne and Mendel XXX mixture, as modified by Wesson ('32), and as further modified by eliminating its contents of calcium and phosphorus.

Calcium was included in the rations in the form of tricalcium phosphate, and additional phosphorus to furnish a calcium-phosphorus ratio of 1:1 was added in the form of dibasic potassium phosphate. Enough calcium was added to each ration so that a daily food intake of 30 Calories furnished 25 mg. of calcium. This level was chosen as being safely

TABLE 1

Composition of rations.

COMPONENTS	RATION			
	1	2	3	4
	%	%	%	%
Dextrin	70.82	58.27	39.89	16.08
Casein	18.88	21.28	24.82	29.39
Oleo oil	4.72	14.04	27.74	45.46
Celluflour	1.89	2.13	2.48	2.94
Salt mix	2.83	3.19	3.72	4.41
$Ca_3(PO_4)_2$	0.86	1.00	1.17	1.40
$K_2H PO_4$	....	0.09	0.18	0.32
	100.00	100.00	100.00	100.00

below the optimum, which is approximately 30 to 50 mg. per day in the young growing rat, and at the same time not low enough to cause a body demand strong enough to mask any effect of the fat on the calcium utilization.

The fat used in preparing the rations was a high-grade oleo oil, with a melting point of about 41.5°C. This fat was chosen because of its rather slow absorption rate, as compared with other fats (Steenbock, Irwin and Weber, '36). It was thought that a slow rate of absorption might conceivably enhance any effect which a fat might have on the utilization of calcium.

Vitamin-free casein was included in all four rations in excess of 19%, and provided equal amounts of protein in equicaloric portions of each of the four diets.

A purified dextrin was used as the carbohydrate. Dextrin was selected in preference to other carbohydrates because it has not been found to exert any influence on calcium retention.

The vitamin supplement, which was the same in equicaloric amounts of all four rations, supplied a daily average of 20 µg. beta-carotene, 25 I. U. irradiated ergosterol, 35 µg. thiamine hydrochloride, 35 µg. riboflavin, 35 µg. B_6 hydrochloride, 20 µg. calcium pantothenate, and 11 mg. of lecithin, which was included for its content of choline.

Determination of intestinal acidity

In order to determine the acidity of the intestinal contents that existed during the experimental period several precautions were taken. A time interval of 5½ hours was allowed to elapse between feeding and killing each rat, and quadruplets were fed at a specific hour for 3 days previous to the determination.

After a rat was killed in the gas chamber at the designated time, the entire gastrointestinal tract was carefully removed and placed between two layers of wet gauze to keep it moist. The duodenum was then removed and its contents expelled into the 0.5 ml. beaker of the Beckman pH meter. Three drops of distilled water were added and mixed thoroughly with the sample before inserting the electrodes and determining the pH value. This value was then checked by remixing and redetermining. The glass electrode was used for all pH determinations. A 4-inch section of the jejunum (6 inches below the duodenum) was removed next, and the contents treated similarly. The third determination was made on the contents of a 4-inch section of the ileum just above the cecum. The acidity of the stomach and cecum was determined last.

The slight dilution of each sample was necessary to increase the volume so that it covered the bulb of the glass

electrode when the 0.5 ml. beaker was used. Such a dilution did not affect the hydrogen ion concentration.

DISCUSSION OF RESULTS

The original plan of the experiment was to feed different diets in equicaloric amounts to each rat in a set of four litter mates, but after 10 days it became apparent that the high-fat ration was severely limiting the consumption of the other rations. Hence it was decided to feed the high-fat diet ad libitum, and to feed the other three diets as planned.

The rats which received equicaloric amounts of the 5, 15 and 28% fat rations grew rapidly at equal growth rates, but the rats receiving the smaller caloric intake of the 45% fat diet ad libitum grew at a slower rate. This was particularly true of rat no. 20 which weighed 52 gm. at the start of the experiment, reached a maximum weight of 61 gm. after 10 days, and then remained nearly constant in weight for the remainder of the 40-day collection period. During the collection period it consumed 17 Calories of the 45% fat ration per day. After the collection was completed, rat no. 20 was fed 17 Calories of the 5% fat diet daily, and proceeded to gain weight at the rate of 1 gm. per day during a 12-day test period.

A consideration of this trial leads to the conclusion that the high fat content of the 45% fat diet exerted a definite depressive effect on the growth rate as well as on the food consumption. It was noted that the rats on this diet excreted a larger number of fecal pellets than did the rats on the other three diets.

The average values for calcium utilization in per cent, summarized in table 2, from the 5, 15, 28 and 45% fat diets, were 80.0, 77.7, 74.1 and 46.8, respectively.

The statistical significance of these values was calculated by Love's modification of "Student's" method ('24). The calculated odds that significant differences exist between the average percentage values for calcium utilization are as follows: 5 and 15% fat, 5:1; 5 and 28% fat, 76:1; and 5 and 45% fat, 5000:1.

TABLE 2

Average calcium utilization and digestibility of fat during 40 days. Six animals on each ration.

RATION	CALCIUM					FAT			
	Intake	Excretion		Reten-tion	Utiliza-tion	Intake	Excre-tion	Digested	
		Urine	Feces						
	mg.	*mg.*	*mg.*	*mg.*	%	*gm.*	*gm.*	*gm.*	%
1	765	16	137	612	80.00	12.20	1.33	10.87	89.10
2	739	15	150	574	77.67	30.20	1.12	29.08	96.29
3	761	18	179	564	74.11	50.18	0.96	49.22	98.09
4	709	17	360	332	46.83	59.88	1.30	58.58	97.83

It is apparent that the utilization of calcium decreased moderately and consistently in the order of the increase in the fat content of the diets from 5% to 28%, and that the utilization from the 45% fat diet was considerably less efficient. These results compare favorably with the work of Knudson and Floody ('40) who noted a similar superiority of 5% as compared with 10% or 20% of fat in the diet in the healing of rickets under the influence of vitamin D.

A study of the intestinal acidity, summarized in table 3, shows that a more acid condition in the intestine resulted from the 5% fat diet than from the diets richer in fat. The average pH values of the contents of the jejunum, in which the greater part of the calcium absorption takes place, were 5.81, 6.02, 6.18 and 6.65, for the 5, 15, 28 and 45% fat diets, respectively. The calculated odds that these values are statistically significant are as follows: 5 and 15% fat, 6:1; 5 and 28% fat, 40:1; and 5 and 45% fat, 434:1.

TABLE 3

Average pH values for intestinal acidity, expressed after 40 days on various fat intakes.

RATION	NO. OF ANIMALS	STOMACH	DUODENUM	JEJUNUM	ILEUM	CECUM
1	7	3.52	5.74	5.81	7.10	6.59
2	7	4.03	5.72	6.02	7.17	6.66
3	5	3.49	5.61	6.18	7.24	6.76
4	6	3.90	6.17	6.65	7.31	6.95

The utilization of calcium paralleled the acidity of the intestinal tract, the most efficient calcium utilization accompanying the most acid reaction. This lends emphasis to the theory of Boyd, Crum and Lyman ('32) that a moderate amount of fat in the diet favors the absorption of calcium by maintaining a favorable acidity in the intestine. This theory does not explain, however, the increasingly poorer utilization of calcium, and the accompanying decrease in acidity, in the order of the increase in fat from 5% to 15, 28 and 45%.

The most efficient calcium utilization was obtained when the diet contained 1 gm. of fat to 0.063 gm. of calcium, and the utilization of calcium decreased as the fat-calcium ratio increased. These results agree with the work of Hickmans ('24) who found that the absorption of calcium by children was most efficient when the food contained 1 gm. of fat to 0.04–0.08 gm. of calcium.

Klinke ('28) studied the solubility of calcium-fatty acid-bile complexes and noted that calcium soaps formed very soluble compounds with desoxycholic acid. He also found that excluding the bile from the intestines of dogs by means of bile fistulae greatly increased the amount of calcium in the feces. Beznák ('31) continued this work and found that the addition of sodium taurocholate to a diet containing calcium and fat materially increased the absorption of calcium. He stated that bile salts play an important role in calcium absorption.

Verzár ('36) suggested that through the hydrotropic action of bile, bile acids form complexes with the calcium and fatty acids that are soluble in water and that can diffuse through the intestinal wall. He also proposed a similar theory for the absorption of fat: the fatty acids, through the hydrotropic action of the bile, form a bile-fatty acid complex (1 molecule of fatty acid to 3 of bile acid) that is water-soluble and readily absorbable by the intestine.

This hydrotropic action theory has been widely accepted to explain the passage of fat through the intestinal epithelium, and it seems possible that part of the calcium might be ab-

sorbed in a similar manner. The work of Irving ('26) shows that water-soluble, ionized calcium can be absorbed without bile in an acid medium, which would serve to explain the absorption of some calcium that takes place on a fat-free, or on a high-fat diet.

If it is assumed that fat affects calcium absorption through the formation of bile-fatty acid-calcium complexes, a necessary condition would be that enough bile be present both for the formation of these complexes and for the absorption of the major part of the fat. An inspection of table 2 suggests that enough bile was present to produce a nearly complete digestibility of the fat in the high-fat diet, but it is possible that nearly all of the bile was utilized for that purpose and that a minimum remained for the formation of bile-fatty acid-calcium complexes.

The increased alkalinity of the intestine after the feeding of the high-fat diet may be due partly to the removal of the bile acids and fatty acids during absorption of the fat, and partly to an increase in the flow of the bile and pancreatic secretions which are alkaline. This flow is stimulated by the ingestion of fat.

It seems probable from the work of Jones ('40) that vitamin D is necessary in the formation or absorption of such bile-fatty acid-calcium complexes in the intestine. In the absence of this vitamin Jones obtained no increase in calcification from feeding sodium oleate, or the calcium soaps of lard, while Boyd, Crum and Lyman ('32) found that the reverse was true when vitamin D was included in the diet.

The inferior calcium retention that resulted from fat-free diets, reported by several investigators, could be explained on the basis that no fat was present for the formation of bile-fatty-acid-calcium complexes. The decreased acidity noted by Boyd, Crum and Lyman ('32), under such conditions, also seems to be a factor contributing directly to a lowered calcium absorption.

The results of experiments now in progress, using similar techniques to determine the percentage fat intake for optimal calcium retention, will be reported later.

SUMMARY

Excellent growth was obtained from diets containing 5, 15 and 28% fat, but 45% of fat in the diet resulted in less growth and in the excretion of a larger number of fecal pellets.

The utilization of calcium decreased moderately and consistently in the order of the increasing fat content of the diets from 5 to 28%, and then decreased considerably for the 45% fat diet.

A more acid condition in the intestine resulted from the 5% fat diet than from the diets richer in fat. The utilization of calcium paralleled the acidity of the intestinal tract, the most efficient utilization accompanying the most acid reaction.

The most efficient calcium utilization was obtained from the diet containing 1 gm. of fat to 0.06 gm. of calcium; and the efficiency of the utilization of calcium decreased in the order of the increase in the ratio of fat to calcium in the diets.

The data are discussed in relation to various theories advanced to explain the physiological relationship between fat and calcium; and it is suggested that at least two factors are involved: (1) the acidity of the intestinal tract, and (2) the formation of readily absorbable bile-fatty acid-calcium complexes.

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THE INFLUENCE OF DIETARY FAT ON LACTATION PERFORMANCE IN RATS

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A dozen years ago Maynard and McCay ('29) reported that the removal of most of the lipids by solvent-extraction from a grain mixture fed to milking cows, and the replacement of these lipids by an iso-dynamic amount of starch, resulted in a marked loss in milk yield. These results have been confirmed by twelve additional experiments with cows and goats, which are cited and summarized in the two most recent publications (Maynard et al., '39, '41). These publications also refer to several studies which have been made of the physiology involved. Both to ascertain whether the same results would be obtained with species other than herbivora, and also to use a species in which the purified diet technique might be employed, studies were begun with rats. Two experiments are here reported.

In both experiments, the mother rats had been fed upon our stock diet[1] from weaning, as well as through gestation. After the birth of the young, pairs of mothers were chosen which were equal in weight, and from whose litters six young for paired groups of nearly equal body weight could be selected. Whenever possible these paired groups were equalized as to sex. One mother was then fed the high-fat diet, and the other, the low-fat diet, equalizing the calorie intake in accordance with the appetite of the one consuming the

[1] The composition of this diet is given in table 1, since it was used also as the low-fat diet in the first experiment.

least amount. This proved to be the animal on the low-fat diet in practically all cases. The young were weighed daily for 17 days to provide growth records during the period when they were entirely dependent upon mothers' milk, thus furnishing a measure of lactation performance. Weight changes in the mothers were also recorded. At the end of the 17 days the young were killed and analyzed to ascertain whether any differences in growth weight were associated with differences in tissue, especially calories stored. No mothers who bore litters of less than six young were used in the experiments, and if, due to accident or other cause, the entire group of six failed to survive the 17-day period, all records for the pair of mothers concerned were discarded.

Carcass analyses on the 17-day-old young included determinations of moisture, fat and ash. Protein was calculated by difference. Immediately after killing the young by ether inhalation, the gastrointestinal tract was removed from each animal. The combined weight of each litter, after removal of the gastrointestinal tracts, was recorded as the weight of the fresh carcasses, and this value was used in the calculations of carcass composition. The carcasses were frozen and then ground preparatory to drying. The drying was carried out in vacuo at 97°C. under an atmosphere of CO_2 and was considered complete when the change in weight was not more than 0.1%.

The material here reported as fat is ether extract obtained as follows: The ground and dried carcasses were extracted with a 4 to 1 mixture of absolute ethyl alcohol and anhydrous ethyl ether. The boiling solvent was in direct contact with the dried carcass material. The total extraction time was 16 hours and 2 portions of solvent were used. The alcohol-ether extract was evaporated to near-dryness on a water bath and the final drying was accomplished in vacuo under CO_2. The resulting material was re-extracted with ether, filtered, evaporated to dryness under CO_2 and weighed.

Ash was determined on the dried defatted carcasses which had been pulverized in a power mill. Two-gram samples were ashed at 560° for 20 hours.

First experiment

In the first experiment our stock diet, containing 4.5% fat, was compared with the same diet modified to contain 9.29% of this nutrient. The composition of these diets is given in table 1. It is seen that the modification consisted of the substitution of 7% of corn oil (crude) and 1% of casein for 8% of the corn meal. The data for calorific value shown in the table were calculated by the use of the factors: 4 Cal. per

TABLE 1

Composition of diets.

NATURAL FOODS (EXPERIMENT 1)			PURIFIED INGREDIENTS (EXPERIMENT 2)		
Constituents	Low-fat diet	High-fat diet	Constituents	Low-fat diet[1]	High-fat diet
				(parts)	*(parts)*
Linseed meal	15	15	Casein	20	20.00
Yellow corn meal	20.875	12.86	Yeast, ether extracted	5	5.00
Ground malted barley	10	10	Cooked starch	40	25.00
Wheat red dog flour	22	22	Sucrose	30	11.25
Oat flour	15	15	Steamed bone meal, ether extracted	2	2.00
Dried skimmed milk	12	12	Hawk and Oser mineral mixture	3	3.00
Soluble blood flour	3	3	Oil	–	15.00
Salt	1	1		100	81.25
Steamed bone meal	1	1	Protein (%)	22.32	27.47
Cod liver oil[2]	0.125	0.14	Fat (%)	0.03	18.46
Casein	–	1	Calcium (%)	0.95	1.16
Corn oil	–	7	Phosphorus (%)	0.71	0.88
	100.000	100.00	Cal. per gram	3.57	4.42
Protein (%)	23.83	22.13	Protein per 100 cal. (gm.)	6.25	6.21
Fat (%)	4.50	9.29	Calcium per 100 cal. (gm.)	0.26	0.26
Cal. per gram	3.30	3.70	Phosphorus per 100 cal. (gm.)	0.19	0.19

[1] To each kilogram of this ration were added 14 mg. calcium pantothenate, 2 mg. pyridoxine and 1 gm. choline hydrochloride.

[2] The cod liver oil used contained 800 A.O.A.C. chick units of vitamin D and 6000 I.U. of vitamin A per gram.

gram for protein and carbohydrate and 9 Cal. per gram for fat. On the basis of the relationship thus obtained, 0.89 gm. of the high-fat diet was fed for each gram of the low-fat mixture. On this basis the intakes of protein and minerals were actually lower on the high-fat diet. Presumably they were not sufficiently lower to make any practical differences, but it was apparent that any differences in the two diets other than the fat tended to favor the low-fat mixture.

The rats with their litters were housed in cages with wire screen bottoms, equipped with feed cups to avoid feed wastage. Any wastage which did occur was accounted for, and adjustments in later intakes were made accordingly so as to keep the actual consumption equalized in terms of calories. The mothers used were approximately 12 months of age and had previously reared several litters successfully. The data for the mothers which reared their six young during the entire 17-day period with substantially equal calorie intakes are presented in table 2. Some additional pairs were removed from the experiment when the death of one of the young spoiled the comparison. A study of the incomplete records of these pairs failed to indicate that these losses of young were any larger on one diet than on the other.

The data for calorie intake in table 2 reveal that success was achieved in equalizing this intake within pairs, the largest difference for any pair being approximately 0.5%. The weights of the paired mothers show a similar degree of correspondence. It is noted that larger differences existed between the weights of the paired litters at the start, as was to be expected. The data as a whole, however, reveal that neither ration had any significant advantage in this respect. For example, the largest difference in favor of the low-fat diet is shown by pair I, and this difference is balanced by one in the other direction shown for pair K.

The data for litter gains show that in thirteen out of fifteen comparisons they were larger for the high-fat diet. They averaged 126 gm. for the latter as compared to 112 gm. for the low-fat diet. As computed by Student's ('25) method,

TABLE 2

Comparisons of low-fat and high-fat diets of natural foods.

PAIR AND LITTER	DIET	CALORIE INTAKE	WEIGHT OF MOTHER		WEIGHT OF LITTER		CARCASS ANALYSIS OF LITTERS				
			Initial	Change	Initial	Gain	Dry matter	Fat	Ash	Protein	Calories
			gm.	gm.	gm.	gm.	%	gm.	gm.	gm.	
A-66	L.F.	1137	314	+13	31.0	118.0	24.5	8.58	4.92	19.8	193
A-65	H.F.	1134	314	—23	33.5	141.5	28.7	17.01	3.80	24.8	301
B-10	L.F.	1214	279	+ 2	34.5	112.5	27.0	12.75	3.26	20.5	236
B-2	H.F.	1212	278	—16	34.5	133.5	27.7	14.84	3.44	24.4	278
C-75	L.F.	1091	245	—23	39.0	104.5	24.8	7.42	3.36	21.7	193
C-74	H.F.	1088	246	—33	35.5	121.5	28.2	13.21	3.61	24.1	261
D-0	L.F.	1203	230	+18	38.5	120.5	25.4	10.22	3.54	22.8	225
D-8	H.F.	1200	227	—13	40.5	140.5	28.5	15.56	4.10	27.4	302
E-2	L.F.	863	264	—15	33.5	87.0	24.0	6.70	2.69	17.2	161
E-40	H.F.	861	261	—48	33.5	93.5	25.4	7.78	2.98	18.6	179
F-69	L.F.	1289	295	+ 6	33.0	136.5	26.6	13.60	3.89	23.3	260
F-31	H.F.	1286	300	—28	38.5	152.5	28.0	17.64	3.92	27.0	319
G-84	L.F.	1175	276	+14	35.0	121.0	25.6	9.99	3.81	22.8	223
G-87	H.F.	1172	271	—11	40.5	132.5	26.5	13.29	3.86	26.0	273
H-62	L.F.	978	276	—41	37.0	102.0	24.4	7.02	3.44	20.8	184
H-9	H.F.	976	292	—43	37.5	126.5	28.7	15.34	3.43	24.6	284
I-74	L.F.	1026	247	—17	47.0	106.0	25.0	8.04	3.75	23.0	206
I-77	H.F.	1031	222	—36	37.0	125.0	28.6	24.44	2.39	16.1	322
J-1	L.F.	873	282	—19	35.5	97.5	24.2	7.39	2.96	18.5	174
J-33	H.F.	871	282	—44	33.0	90.5	23.9	5.97	2.66	18.0	158
K-87	L.F.	838	254	—38	32.0	77.0	23.3	5.37	2.47	15.3	137
K-7	H.F.	836	256	—59	44.0	78.0	25.1	5.80	2.93	19.4	164
L-68	L.F.	1279	315	0	37.5	129.0	27.2	14.18	3.62	23.2	265
L-66	H.F.	1248	316	+ 1	35.0	144.0	26.9	13.22	3.85	25.8	271
M-68	L.F.	1544	311	+10	36.5	163.5	28.6	15.94	4.27	31.8	330
M-69	H.F.	1469	305	+ 7	35.5	166.5	29.1	20.81	*	*	*
N-9	L.F.	1173	280	+ 8	36.0	134.0	26.6	13.80	3.64	23.7	264
N-1	H.F.	1144	291	— 9	33.0	132.0	28.3	15.08	*	*	*
O-70	L.F.	870	289	—45	41.5	68.5	23.2	4.75	2.68	16.0	135
O-32	H.F.	868	299	—67	38.0	112.0	25.7	8.99	*	*	*

* Carcass samples lost before data obtained, due to an explosion.

the odds in favor of the results being significant are 1249:1. The daily weight records (not shown) indicated that the more rapid growth of the litters from the mothers receiving the high-fat mixture became evident beginning at about the eighth day. That the gains in body weight reflected actual differences in tissue substance is indicated by the data for the percentage of dry matter in the carcass. These data show that in thirteen out of fifteen cases the young reared on the high-fat diet contained a larger percentage of dry matter, with calculated odds greater than 4999:1 in favor of this result. The evidence here given is further borne out by the other data for carcass composition. In thirteen out of fifteen comparisons, the young from the mothers receiving the high-fat diet contained more fat, the litters on this diet averaging 13.9 gm. compared to 9.7 gm. for the low-fat diet. The odds in favor of the significance of the difference are 356:1. The figures for protein favor the high-fat diet in ten out of twelve cases for which the data were obtained. Here the odds were 27:1. The figures for ash are variable and show no significant trend, although they favor the high-fat mixture in the majority of the cases.

The calorific values of the carcasses were calculated to give a single measure of the differences in energy stored on the equi-caloric intakes. These values were obtained by multiplying grams of carcass fat by 9.45 and grams of protein by 5.65, representing the average heats of combustion of these nutrients. The data reveal an advantage for the high-fat diet in eleven out of the twelve comparisons for which data were available for the calculation. The odds in favor of the significance of this difference are 999:1. On the average the litters from the mothers receiving the high-fat diet contained approximately 25% more calories than their pair mates.

Certain animals were used twice in experiment 1, being placed first on one diet and then on the other. A-66, which received the low-fat diet and performed less satisfactorily in all respects than her pair mate, later received the high-fat diet as L-66 and excelled her pair mate. Similarly B-2, which excelled on the high-fat diet, proved inferior to her pair mate

when later, as E-2, she received the low-fat mixture. Insofar as the data were obtained the same was found true for the animal which appears once as G-87 (high-fat) and again as K-87 (low-fat). These comparisons suggest rather clearly that the uniformly better performance on the high-fat diet could not have been due to the chance selection of animals of higher productive capacity for that diet. The significance of the results is strengthened thereby.

In connection with differences in calories produced, attention should be given to the column of figures in table 2, showing the changes in the weights of the mothers. It is noted that losses in weight occurred in the majority of cases and that some very large losses are recorded. One would expect such losses to be more likely to occur under the limited feeding system imposed by the paired feeding technique. Of more significance, however, the decreases in weight are greater (or the gains are less) for the mothers on the high-fat diet in all cases except one. On the average the mothers receiving this diet revealed an average net loss of 18.6 gm. greater than the mothers on the other diet. This means that whereas the high-fat diet clearly resulted in a larger lactation performance, this performance occurred in part at least at the expense of body reserves. Without knowing the composition of the tissues lost by the mothers, it is impossible to make any estimate of the relationships involved. Citing an extreme case, animal O-32 lost 67 gm. while nursing a litter which gained 112 gm., whereas her pair mate lost 45 gm. nursing a litter which gained 68 gm. Though it lost more body tissue, the much larger production performance of the high-fat mother is evident. The equalized feeding technique does not disclose whether, if the high-fat diet had been fed ad libitum, smaller losses would have occurred accordingly. This question was studied later as is reported under experiment 2. In looking over the data as a whole one notes that for certain pairs the gains of the litters markedly exceeded those of other pairs. These differences are roughly correlated with differences in

calorie intake, and presumably individual appetites are here concerned.

The average weights at 17 days of all the young reared by the mothers on the low-fat and high-fat diets were 24.7 gm. and 27.1 gm., respectively. These figures, particularly the latter, represent excellent weights for the average of a litter of six at 17 days in our colony. They indicate that the comparison was made at a high level of performance.

Second experiment

In this experiment a purified diet designed to be very low in fat (actually 0.03% of ether extract) was selected and modified by the substitution of 15 parts of either crude coconut oil or crude corn oil for an equal amount of starch to provide the high-fat ration. These diets are shown in table 1. The calorific values per gram are calculated as described in experiment 1. On the basis of these calculations the calorific intakes per pair were equalized by feeding approximately 0.8 gm. of the high-fat diet for each gram of the low-fat mixture. It is noted that whereas the high-fat diet had more protein, calcium and phosphorus on a percentage basis, both diets supplied the same amounts per 100 calories. Thus the mothers' intakes were equalized for these nutrients also, a provision which could not be met in experiment 1 without markedly altering the ingredient relations.

Each animal on the purified rations received a daily supplement of 1 drop of A-D vitamin concentrate, so diluted with cottonseed oil that each drop contained 13.8 U.S.P. units of vitamin A and 1.95 units of vitamin D. To insure an adequate supply of vitamin D, each animal received direct irradiation at a distance of 3 feet from a quartz mercury vapor lamp for a period of 5 minutes weekly. It is noted in the table that the low-fat diet was supplemented with choline, pantothenic acid and pyridoxine. The choline was added to make sure that any value obtained from the added fat was not due to this constituent. It was planned, considering the possibility of additional needs, to add the other vitamins to both diets, but due

to an error they were omitted from the high-fat diet. Thus if these added vitamins were of benefit, the low-fat mixture was favored thereby. Despite these additions it cannot be said, particularly in view of the recent work of Sure ('41 a, '41 b), that either diet was optimum in all vitamins required for lactation. It is considered, however, that the content of the known vitamins of the low-fat diet was equal to that of the high-fat diet. Further, it should be borne in mind that the rats received a natural diet up to the time of parturition and that the purified diets were fed for only 17 days. The questions here involved are now under study, as is referred to later.

The general plan of the experiment was the same as for the one previously described. At the outset, females approximately 1 year of age which had previously produced several litters were used, but it was found impossible to obtain satisfactory food consumption, or even litter survival by shifting them suddenly to the purified rations following parturition. After failures with approximately fifteen of these females, young females about 90 days of age were used and successful comparisons were obtained. The data are presented in table 3 for eleven pairs of females. For five of these pairs the high-fat ration contained coconut oil and for the others it contained corn oil. Both oils were used in order to ascertain whether the wide differences in fatty acid distribution here represented might be reflected in differences in lactation performance.

It is noted in table 3 that for each pair, success was achieved in selecting mothers of comparable weight and in equalizing their calorie intakes. It is also noted that any variations in the initial weights of the litters were less than in experiment 1. The gains of the litters are in favor of the high-fat diet in ten out of eleven cases, by odds of 73:1, averaging 122 gm. for the low-fat diet and 138 gm. for the other. The differences here involved represent an actual increase in dry matter as is indicated by the percentage figures for the latter. The odds here are 1249:1. In all comparisons the high-fat diet resulted in more fat in the litters, the litters on the high-fat diet

TABLE 3

Comparisons of low-fat and high-fat purified diets.

PAIR AND LITTER	DIET	CALORIE INTAKE	WEIGHT OF MOTHER		WEIGHT OF LITTER		CARCASS ANALYSIS OF LITTERS				
			Initial	Change	Initial	Gain	Dry matter	Fat	Ash	Protein	Calories
			gm.	*gm.*	*gm.*	*gm.*	%	*gm.*	*gm.*	*gm.*	
A-59	L.F.	1196	187	+12	31.5	91.0	28.3	11.91	2.76	16.7	207
A-8	H.F.[1]	1196	190	— 5	31.0	119.5	28.0	13.80	4.46	20.0	243
B-13	L.F.	1257	231	+ 2	37.5	134.5	26.1	12.03	4.30	25.5	258
B-12	H.F.[1]	1260	237	—14	35.5	146.5	33.2	25.69	3.81	25.5	387
C-17	L.F.	1190	201	+ 9	34.5	109.5	25.2	9.76	3.18	20.2	206
C-4	H.F.[1]	1183	194	— 1	35.0	121.0	28.7	14.88	3.42	22.2	266
D-14	L.F.	1385	240	0	33.5	149.5	29.8	19.14	4.17	26.2	329
D-17	H.F.[1]	1389	236	—10	33.5	174.0	32.5	26.20	4.32	32.0	428
E-3	L.F.	816	220	—30	37.5	94.0	23.8	7.33	3.03	18.2	172
E-1	H.F.[1]	818	228	—28	39.5	107.5	27.3	11.85	3.21	21.2	232
F-29	L.F.	1498	242	+44	36.5	132.5	28.3	16.90	3.51	22.3	286
F-3	H.F.[1]	1501	250	0	37.0	173.0	30.8	23.12	4.31	32.5	402
G-16	L.F.	1519	216	+21	32.0	158.0	28.7	17.27	4.19	28.1	322
G-12	H.F.[2]	1523	216	— 8	34.5	141.5	30.3	19.48	4.07	24.6	323
H-61	L.F.	1128	206	— 2	30.5	110.5	25.7	9.76	3.37	20.0	205
H-19	H.F.[2]	1131	261	—32	35.0	116.5	29.3	16.09	3.38	21.7	275
I-19	L.F.	1014	264	—34	32.5	80.5	26.2	8.08	2.61	16.0	167
I-13	H.F.[2]	1017	261	—42	35.5	125.5	27.6	13.07	*	*	*
J-59	L.F.	1349	236	— 6	37.0	152.0	28.9	16.95	*	*	*
J-16	H.F.[2]	1353	250	—15	35.0	155.0	30.2	21.52	3.92	26.7	354
K-17	L.F.	1291	230	0	38.5	135.0	27.3	13.63	3.73	25.6	273
K-18	H.F.[2]	1294	233	—17	34.0	141.0	30.8	20.67	3.55	25.1	337

[1] Fat supplied by corn oil.
[2] Fat supplied by coconut oil.
* Carcass material lost before data obtained.

averaging 18.8 gm. and those on the other, 13 gm., the odds being over 4999:1. This is true for protein in six out of the nine cases for which data are available, one pair showing no difference in either direction. But here the odds are only 9.5:1. The ash data favor this same diet in six out of nine cases. The figures for calories favor the high-fat ration in all comparisons, with odds of 1249:1. On the average, the litters from the mothers receiving the high-fat diet contained approximately 28% more calories than those from the other mothers. The figures do not reveal any significant differences between the performances of the mothers on the high-fat diet where the oils were different. A more critical experiment would be needed to demonstrate any differences which may exist.

The young from all the mothers receiving the low-fat diet averaged 26.1 gm. body weight at 17 days, and those produced on the high-fat mixture averaged 27.4 gm. Like the first experiment, these data show that the comparison was made at a high level of performance.

Also like the first experiment, the increased lactation performance on the high-fat diet was accompanied by a larger loss (or a lesser gain) in the mothers' reserves, as is shown by the data for change in their weight. On the average the mothers receiving the low-fat diet gained approximately 1 gm. while those on the high-fat mixture lost 16 gm. In order to ascertain whether this loss was due to the restricted feeding, five mothers with new born litters of six were placed on the high-fat (corn oil) diet and fed ad libitum for 17 days. The results were as follows:

MOTHER NO.	WEIGHT CHANGE OF MOTHER	AV. WEIGHT OF YOUNG AT 17 DAYS
	gm.	*gm.*
49	+ 7	33
181	+14	34
131	+12	33
141	+20	35
97	+ 4	39

These data show that with ad libitum feeding the high-fat diet both caused the mothers to gain in weight instead of lose and to surpass the previous performance for milk production. The latter is indicated by a comparison of the data for average weight of young at 17 days with the average figure of 27.4 gm. obtained with the restricted feeding. Clearly the results produced with the high-fat diet fed ad libitum represent a remarkable lactation performance.

The similar results of two experiments with different diets furnish very strong evidence that the substitution, on an isodynamic basis, of fat for a part of the carbohydrate in a low-fat diet results in a higher lactation performance. This is in accord with the previous findings of this laboratory with cows and goats. Various possible explanations, such as more efficient metabolism of fat calories for fat secretion, need for specific fatty acids, vitamin relationships, and others, have been discussed in previous reports. The present experiment provides no answer here other than revealing no significant difference between two fats of widely different fatty acid distribution. The fact that the larger lactation performance in the rats fed the diet of higher fat content was accomplished in part at least at the expense of the mothers' reserves might suggest some specific stimulating action. The need is recognized for a repetition of the work with more attention to all of the vitamins required for lactation in the rat. Specifically it is possible that on the low-fat diet there was a relative shortage of thiamine for the metabolism of its higher carbohydrate content. Calculations suggest that this possibility is an unlikely explanation of the results obtained but an experiment to test this question is now in progress.

While the differences in favor of the high-fat diet appear convincing and while no evidence of loss of the mothers' reserves was noted with ad libitum feeding despite a further increase in lactation performance, these results by themselves should not be interpreted to apply to other species. The very

different composition of rat milk, particularly its fat content, must be kept in mind. The principal contribution of the present experiment is to provide a technique by which the various physiological questions can be studied to better advantage than in the cow or goat. Such studies are being planned.

SUMMARY

Paired-feeding studies, involving equalized calorie intakes, are reported in which lactation performance was measured by the growth of standardized litters and by their composition. In one experiment a diet of natural foods containing approximately 4.5% fat was compared with a similar diet containing approximately 9% fat. The young from the mothers on the high-fat diet made better growth and contained more dry matter and fat in thirteen out of fifteen paired comparisons. Similar results were obtained for protein and calorie content. In a second experiment in which purified diets containing 0.3% and 18% fat were compared the data for the high-fat diet indicated a superiority in growth and in dry matter content in ten out of twelve comparisons, in fat and calorie content in all cases, and in protein in six out of nine cases. A statistical analysis indicated a high degree of significance for the results of both experiments. With the limited feeding practiced, the better lactation performance on the high-fat diet occurred in part, at least, at the expense of the mothers' reserves.

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ASCORBIC ACID EXCRETION AT KNOWN LEVELS OF INTAKE AS RELATED TO CAPILLARY RESISTANCE, DIETARY ESTIMATES, AND HUMAN REQUIREMENTS [1]

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INTRODUCTION

The literature concerning the significance of vitamin C in human nutrition has been reviewed by Bessey ('38), King ('38), Smith ('38) and Todhunter ('40). Several types of data have been used as a basis for estimating requirement including (1) urinary ascorbic acid, (2) capillary fragility, and (3) ascorbic acid in blood, all at different levels of intake and under a variety of conditions. Kellie and Zilva ('39) reported that a minimum intake of 30 to 40 mg. of ascorbic acid is necessary to produce a standard response to a "saturation" dose of 600 mg. ascorbic acid. Belser, Hauck and Storvick ('39), using a 400 mg. saturation dose, found that five women subjects required 70–100 mg. and two men needed 110–135 mg. of ascorbic acid intake daily to maintain saturation. Seventy to 90 mg. of ascorbic acid was necessary to keep three women saturated according to Todhunter ('40). Ralli, Friedman and Sherry ('39) observed no clinical symptoms of vitamin C deficiency in three men on a 50 mg. intake but observed no increase in urinary excretion until the intake reached 100 mg. and the blood plasma level reached 0.1 mg.

[1] Contribution no. 607 of the Rhode Island Agricultural Experiment Station.

[2] A portion of the data was presented by Miss Tatiana Levcowich in partial fulfillment of the requirements for the degree of Master of Science at Rhode Island State College, June, 1939.

A study of the urinary ascorbic acid excretion and capillary resistance of college women (Mitchell, Merriam and Batchelder, '38) has led to further investigation at Rhode Island State College of the vitamin C status and ascorbic acid requirement of such women.

EXPERIMENTAL

Student diets and excretions-survey study. A 1938 study of Rhode Island State College women, for whom results for 1937 have already been reported (Mitchell, Merriam and Batchelder, '38), showed an average urinary ascorbic acid excretion from thirty-two women of 24 mg. (range 5–122 mg.) as compared with 17 mg. (range 5–56 mg.) for the same subjects the previous year. The 1938 average ascorbic acid intake was 122 mg. (range 56–253 mg.) as compared to 81 mg. (range 32–126 mg.) in 1937, when calculated from a dietary record for the 7 consecutive days preceding and including the 24-hour urine collection. The coefficient of correlation of the intake and output of ascorbic acid for all 1938 studies was +0.48. This was slightly higher than that for the previous year. The coefficient of correlation of the urinary ascorbic acid excretions with that of the same subjects tested the year before and already reported was +0.45. Although the absolute values of the calculations of intake are open to question as discussed later, increased urinary output shows a consistent relation to increased intake.

Studies were made on the urinary ascorbic acid excreted by eight women under various conditions: (1) when diets were freely chosen; (2) when ascorbic acid intake was increased through ingestion of large quantities of citrus fruits and vegetables; (3) when ascorbic acid in the diet was reduced to a minimum; (4) when massive doses of crystalline ascorbic acid were given; and (5) when ascorbic acid was given at various test levels for determination of dietary needs. Capillary resistance tests were made while the subjects were on different levels of known ascorbic acid intake.

The college medical office classified these women, through medical examination, x-ray, and urine analysis, as healthy and capable of carrying on the usual college activities.

The methods for determining ascorbic acid and capillary resistance were the same as those previously described for the Rhode Island studies by Mitchell, Merriam and Batchelder ('38). For the last two subjects on whom most of the studies have been made, diets averaging not more than 5 mg. ascorbic acid were prepared in a special kitchen and were planned to meet present dietary standards for proteins, minerals, and vitamins. In order to assure optimal intakes of the vitamins

TABLE 1

Urinary excretion of ascorbic acid as related to estimated intake from freely chosen diet and to intake of crystalline ascorbic acid.

NO. OF DETERMINATIONS	INTAKE ESTIMATED		EXCRETION	
	Average	Range	Average	Range
	mg.	*mg.*	*mg.*	*mg.*
29	87	27–193	13	6–16
27	121	44–253	22	18–29
	Intake crystalline ascorbic acid			
	50 [1]		22	17–29

[1] Plus 5 mg. in foods included in the diet.

A, D, and the B complex, dry yeast and halibut liver oil were given daily. For the earlier subjects, the diets were planned on the basis of foods available at the college cafeteria and supplemented with yeast and haliver oil as mentioned above. The results indicate that this arrangement provided a satisfactory vitamin C free diet.

Excretion at different intake levels. Excretions of subjects at a 50 mg. intake level are used to interpret the results of the student diet studies as shown in table 1. The excretion of the crystalline ascorbic acid group was measured during a post-saturation period. The excretion of the survey group was measured at the customary level of intake and is pre-

sumably characteristic of that intake. Even though daily fluctuation may be a source of error in individual cases, the results for the whole group are probably representative.

The members of the survey group never excreted as much ascorbic acid as the group receiving 50 mg. daily in a post-saturation period. This indicates that their habitual daily intake (although high according to calculation) was actually quite low.

The evidence leads us to conclude that excretions on known intakes of 50 mg. crystalline ascorbic acid plus an average of 5 mg. taken in the basal diet are comparable to excretions on freely chosen diets which, by calculation, would appear to contain at least twice as much as the amount excreted. Most of the subjects on the freely chosen diets were eating in a college cafeteria. Comparison of excretions on a known intake and on a calculated intake indicates that there is danger of overestimating the actual vitamin C content of diets, particularly where food is prepared and served under conditions which may contribute to the destruction of vitamin C. From a practical standpoint, it may be desirable, therefore, to publish popular food guides which are specific as to the kind and amount of vitamin C food required. Important factors such as the length of time between harvesting and eating, storage conditions, and methods of cooking may reduce the vitamin C in a food to a point far below that shown in the most extensive of tables.

Capillary fragility as related to character of diet. In the light of new reports concerning capillary fragility as affected by vitamin P as well as vitamin C, capillary resistance tests (Dalldorf, '33), were resumed when subjects M. A. and R. B. were observed at various known levels of crystalline ascorbic acid intake (table 2).

An average of at least 15–20 cm. negative pressure was necessary to produce petechiae on the front of the arm for both subjects. A similar average was obtained in the group studied in 1937. Capillary resistance was not significantly

affected by varying the amount of crystalline ascorbic acid added to the vitamin C-free diet. The capillary resistance values on the back of the arm were, however, consistently higher in both subjects. The average was 30–35 cm. negative pressure. This differs from the results reported by Anderson,

TABLE 2

Average daily ascorbic acid excretion at different levels of intake as related both to excretion following a 400 mg. dose and to capillary resistance.

SUBJECT	ASCORBIC ACID INTAKE	EXCRETION		CAPILLARY RESISTANCE [2]			
				Front of arm		Back of arm	
		During test period [1]	Following 400 mg. dose	Test period	After 400 mg.	Test period	After 400 mg.
	mg.	*mg.*	%	*cm.*	*cm.*	*cm.*	*cm.*
M. A.	40	18	16	10	15	15	20
	50	24	28	15	15	40	40
	50	20	33	20	20	35	35
	110	62	42	20	15	40	40
	120	33	30	15		40	
R. B.	40	19	26	10	15	20	25
	50	22	28	15		25	
	50	23	18	15	15	30	30
	60	34	27	15	15	30	30
	70	32	24	15	20	30	30
	90	55	32	15	15	35	30
	110	41	43	20	15	30	35
	120	50	32	20	20	40	30
	130	73	47	25	25	40	40
	140	71	21	25		40	
	140	69	57	20		30	

[1] Average of last 3 days of each 6-day test period.
[2] Negative pressure necessary to produce petechiae.

Hawley and Stephens ('36) who found only 1–2 cm. greater negative pressure necessary for the outer arm as compared to the inner arm.

In the nineteenth week of experiment the petechiae resulting from the test, although appearing at the same pressure and in the same numbers, began to increase in size and were so much

larger than those previously observed that the subjects made comments regarding the change. The petechiae of subsequent tests continued to remain large until the Easter recess, during which the subjects returned to freely chosen diets high in vitamin C rich foods. Upon the return to Campus when M. A. was tested the petechiae had returned to smaller size. Although no test could be made on R. B. until 4 days after she had returned to the basal diet plus crystalline ascorbic acid, similar, but not as striking, results were observed. About 2 weeks later the petechiae began increasing in size again.

These observations are similar to those of Scarborough ('39) who reported that conflicting evidence by previous observers might be accounted for by the fact that vitamin P, as well as vitamin C, affects capillary fragility. Although our evidence is limited, it seems possible that the large petechiae of subjects on our vitamin C free diet plus crystalline ascorbic acid might be evidence of lack of another factor ordinarily ingested when the vitamin C of the diet is taken in the form of natural foods. In our two subjects a few days on natural foods appeared to result in smaller petechiae. This may be evidence of a lack of vitamin P in the foods included in the vitamin C free diet. Further study is needed in this connection.

Excretion on vitamin C-free diet. Table 3 summarizes the urinary ascorbic acid data of three subjects on the vitamin C deficient diet (average ascorbic acid content, 5 mg. daily),

TABLE 3

Ascorbic acid excretion on a vitamin C-free diet.

SUBJECT	DAYS ON DIET	DAILY EXCRETION	
		Average	Range
		mg.	*mg.*
T. L.	1	36	
	2– 8	13	11–16
	9–13	10	9–11
C. B.	1	16	
	2– 9	11	7–16
M. A.	1	26	
	2– 7	16	12–19

after long continued high intake. Excretion for the first 24 hours on the deficient diet fell sharply as compared to that for the previous day. By the second day, the excretion had fallen to a characteristic resting level similar to that reported by O'Hara and Hauck ('36).

During the first day subjects T. L., C. B., and M. A. excreted 36, 16, and 26 mg. respectively; while during the following periods of 7, 8, and 6 days respectively the excretions averaged 13, 11, and 16 mg. During an additional 5 days on the diet, T. L. excreted an average of 10 mg. Apparently the exact level of previous vitamin C intake has little effect on the excretion after 1 day on a diet deficient in this factor. The apparent vitamin C content of the urine, however, is maintained at a somewhat higher level than the intake, perhaps because appreciable amounts of other reducing substances are included in this figure. This was suggested by Ralli, Friedman and Sherry ('39), whose results by titration were slightly higher, and by photoelectric colorimeter somewhat lower than ours. It may, however, be due in part to a continuous loss of vitamin C from the tissues.

Excretion as indicative of requirement. The records for subjects M. A. and R. B. at different levels of intake following "saturation" as interpreted by O'Hara and Hauck ('36) are summarized in table 2. After the end of each test level the subjects were fed high vitamin C, usually 200 mg., until a 50% excretion of a 400 mg. dose was observed. M. A. tended to take longer to reach this "saturation" level after each test period than did R. B. For that reason it was not possible to test her at all levels observed for R. B. during the same period of time. The excretion at increasing levels of intake showed a tendency to rise, but neither these figures nor the percentage excretion of a 400 mg. dose following each period on a test level showed sufficiently consistent and regular increments to justify a conclusion that the higher levels were of specific advantage. Ralli, Friedman and Sherry ('39) raised the question, in connection with blood plasma levels, whether intakes necessary to give maximum value for ascorbic

acid content of blood and tissues are essential even though they may be desirable. We have no subjective nor objective evidence of ill-effects either at the lowest or at the highest levels of ascorbic acid fed. There is no evidence that the body, after a period on low as compared with high ascorbic acid intake, needed to accumulate more total ascorbic acid before saturation was reached.

Subjects M. A. and R. B. had a basal energy metabolism, 10 and 17% respectively, below the Aub and Dubois standards as modified by Boothby and Sandford. In the light of the close relation of ascorbic acid to oxidation-reduction processes in the body, it seems reasonable to assume that an individual's requirement may vary with the total energy metabolism. It is possible also that a slight additional loss may be due to excessive perspiration resulting from heat or unusual activity as reported by Bernstein ('37). On the basis of these observations and in the light of other less complete studies, it seems safe to conclude that the minimal ascorbic acid requirement for fairly active college women may lie at about 50 mg. daily. A dietary standard or allowance for ascorbic acid estimated with the safety factor of 50%, commonly used for many nutrients, would then be 75 mg.

As previously discussed, this figure is based on studies with crystalline ascorbic acid. If it is to be applied to estimates of intake on freely chosen diets it should probably include only foods such as freshly expressed citrus fruit juices, freshly boiled or baked potatoes, and other products not likely to have suffered serious losses of vitamin C due to local differences in variety or method of preparation. An example of such differences is reported by Murphy ('41).

SUMMARY AND CONCLUSIONS

Certain aspects of vitamin C metabolism have been studied in forty-five young women during a survey study and during nine intensive control periods. Capillary fragility and urinary ascorbic acid, as observed when the ascorbic acid intake was estimated from freely chosen foods and when crystalline

ascorbic acid was added to a low vitamin C diet at various known levels, have been reported and discussed. Conclusions based upon observations made during the investigation are:

1. The calculated ascorbic acid content of the foods in a freely chosen diet tends to be overestimated, and may lead to a false sense of security as to vitamin C intake, unless very specific food guides can be set up.

2. Capillary resistance is not significantly affected by variation in known crystalline ascorbic acid intake. More negative pressure, however, is necessary to produce petechiae on the back of the arm than on the front. Over an extended period on a crystalline ascorbic acid intake the petechiae become larger in size, but do not change in number. This suggests that the vitamin C-free diet used here may lack vitamin P or some other factor affecting capillary fragility.

3. Urinary ascorbic acid excretion during a vitamin C deficient diet drops sharply on the first day, and on the second day reaches a fairly constant resting level for all subjects.

4. For moderately active young women a minimal requirement of 50 mg. and a dietary standard (or allowance) of 75 mg. daily appears satisfactory.

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STUDIES OF LIBERAL CITRUS INTAKES. I.[1]

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The literature affords several instances of attempts to study the tolerance of the human body for fruit juices rich in citric acid. In many of these (for example, Blatherwick and Long, '22; Saywell and Lane, '33) the amount of citric acid which escaped oxidation was computed indirectly from the "total organic acids" of the urine, on the presumption that the rise in this value when citrus juices are taken is wholly due to, and fully reflects, any "extra" excretion of "unburned" citric acid. That both aspects of this assumption are unjustified was demonstrated in an earlier study (Sherman, Mendel and Smith, '36 b), by direct determination of citric acid side-by-side with titration of "total organic acids" under various conditions.

However, in the carefully controlled studies of Schuck ('34) on six young women given 1000 cc. daily of orange juice, the urinary excretion of citrate was determined directly during both control and experimental periods, each of several days' duration. In each case as reported by Schuck, the excretion of citric acid was greater on the days when orange juice was taken, the increase of daily elimination for her different subjects ranging from 0.234 gm. to 1.294 gm. of citric acid, and corresponding to 2.6 to 16.6% of the citric acid contained in the fruit juice (7.95 to 8.51 gm.). With the diet maintained constant otherwise, orange juice ingestion invariably de-

[1] The aid of a grant from the Florida Citrus Commission is gratefully acknowledged.

creased the acidity of the 24-hour urine sample of Schuck's subjects, the change averaging 0.9 pH unit.

With the greatly increased supply of citrus fruits during the past few years, and the growing tendency to commend them to an ever more prominent place in the dietary, it seemed of importance to determine: (1) whether in a larger representation of the population a considerable proportion would be found to show such a relatively poor utilization of dietary citrate as the one of Schuck's subjects who eliminated one-sixth of the ingested citric acid unchanged; and (2) whether at still higher levels of consumption of citrus juices any indication of intolerance would become evident, either in subjective sensations or in the chemistry of the urine, by a greatly increased output of citric acid or a lowered pH value.

EXPERIMENTAL

The subjects of these experiments on grapefruit juice were nine young women and sixteen young men, all between the ages of 22 and 37 years. On the so-called control day, each of these subjects collected, under toluene and with the customary precautions, the 24-hour urine sample. On the following ("experimental") day, during the first 10 hours of which the subject drank 1800 cc. of fresh grapefruit juice, a second 24-hour sample was collected. A record was kept of the amounts and kinds of food consumed on both days, and from this the citric acid intake (other than from the grapefruit juice) was estimated from the figures of Hartmann and Hillig ('34). The subjects were provided with a list of foods of high citric acid content, which they were requested to avoid; but in some instances milk was taken, despite its considerable citrate content. Each of the ten subjects indicated by footnote 1 in table 1 had an identical basal diet on the control and experimental days. The basal diets of the various individuals were, however, different.

The grapefruit juice was freshly extracted for each experiment and strained through a coarse sieve. Each batch was analyzed for total citric acid by the method of Pucher, Sherman and Vickery ('36). The thirteen different samples

of grapefruit juice used in this series showed an average content of total citric acid of 1.20%, with a range from 0.82 to 1.70%. The five samples which were of the "seedless" variety had a lower citric acid content (1.01%) than did the eight samples with seeds (1.32%), obtained at the same seasons of the year.

On each 24-hour urine sample, the pH was measured, using the glass electrode[2], and the total citric acid (i.e., sum of free acid and salt forms) was determined by the method of Pucher, Sherman and Vickery ('36).

In addition to serving as subjects in this type of experiment, four individuals on another day consumed their same basal diet but, instead of drinking the grapefruit juice over a 10-hour period, took a large volume all within 15 minutes. Urine was collected during a control period of 2 hours immediately before the juice was drunk, and thereafter in four successive 1½-hour intervals, followed by a 16-hour period, each of these six samples being analyzed separately for both pH and citric acid content. The findings of these short-time experiments are to be described more fully later, and are mentioned only briefly in the present discussion.

RESULTS AND DISCUSSION

Effect of grapefruit juice on citrate excretion. In table 1 is shown for each subject the total citric acid intake on the control and on the experimental day, the 24-hour urinary excretion of citrate on the 2 days, and the difference between the latter two values expressed as a percentage of the difference between the former two values. This last-mentioned value may be regarded as representing the percentage of the extra citric acid ingested in the grapefruit juice which escaped oxidation in the body[3], if it may be assumed that the basal or

[2] All measurements of pH were made by Dr. Oscar E. Lanford.

[3] The four studies in which urinary citrate was determined in samples collected over successive short intervals of time indicate that, even when a large volume of grapefruit juice is ingested at one time, any resulting extra excretion of citrate ceases within about 6 hours. Since in the experiments summarized in table 1, the 24-hour collection terminated at least 14 hours after the last portion of juice was taken, it seems entirely probable that such samples included all of the unburned citric acid which escaped through the kidneys.

endogenous excretion of citrate was the same on the 2 days. This assumption, is, however, not altogether valid, for Sherman, Mendel and Smith ('36 a) showed that, on a diet

TABLE 1

Data for individual subjects on citric acid intake, urinary excretion of citric acid, and pH of the 24-hour urine sample.

SUBJECT	CITRIC ACID INTAKE		CITRIC ACID EXCRETION		INCREASED EXCRETION OF CITRIC ACID AS % OF INCREASED INTAKE	URINARY pH	
	Control	Exptl.	Control	Exptl.		Control	Exptl.
Males	*gm.*	*gm.*	*gm.*	*gm.*			
C. S. R.[1]	0.48	15.33	0.515	0.792	1.87	6.62	6.73
R. W. L.	1.73	21.84	0.546	1.094	2.72	6.18	7.06
R. G. L.	1.87	22.00	0.299	0.664	1.81	6.46	7.02
W. D.	0.72	16.93	0.420	0.891	2.91	6.46	7.08
H. C. K.	0.26	17.08	0.279	0.391	0.67	5.86	6.06
Z. H.[1]	0.08	19.88	0.237	0.705	2.36	5.62	6.61
R. C. B.[1]	0.29	20.09	0.354	0.676	1.63	5.40	6.12
F. E. H.	1.01	27.91	0.468	0.742	1.02	5.85	6.45
G. R. L.	1.01	27.91	0.610	1.168	2.07	5.91	6.75
E. L.[1]	0.49	17.01	0.653	0.848	1.18	6.00	6.05
F. A. F.	0.47	24.25	0.426	1.040	2.58	5.78	6.70
H. McC.	0.29	23.98	0.293	0.666	1.57	6.29	6.82
D. T. V.	0.58	25.95	0.418	0.741	1.27	5.59	6.52
H. D.	0.48	26.10	0.354	0.805	1.69	5.73	6.52
H. B.	0.50	31.11	0.296	0.818	1.38	5.99	6.74
G. H.	0.12	30.80	0.275	0.738	1.51	5.48	6.70
Mean ± its P. E.	0.65	23.14	0.403± 0.021	0.802± 0.031	1.76± 0.010	5.95± 0.06	6.62± 0.05
Females							
M. C. U.[1]	0.36	21.96	0.731	1.469	3.42	6.22	6.75
A. B. R.[1]	0.42	15.27	0.583	1.008	2.86	6.47	6.81
M. M. W.	1.05	17.35	0.641	1.040	2.45	5.77	6.37
F. V. D.[1]	0.68	29.03	0.528	1.080	1.95	5.71	6.55
A. Z.	0.47	29.21	0.970	1.476	1.76	5.62	6.63
A. U.[2]	0.48	16.86	0.671	1.074	2.91[2] }	6.05[2] }	6.70[2] }
A. U.[2]	0.50	17.59	0.536	1.112	2.97[2] }	5.58[2] }	6.28[2] }
M. L.[1]	0.49	17.01	0.659	0.827	1.20	6.17	6.57
I. M. S.[1]	0.25	24.10	0.638	1.263	2.62	5.55	6.80
C. S. L.[1]	0.04	23.89	0.435	0.771	1.41	6.29	6.38
Mean ± its P. E.	0.47	21.76	0.643± 0.033	1.111± 0.055	2.27± 0.175	5.94± 0.03	6.58± 0.04

[1] Subject had same basal diet on control and experimental days.

[2] Experiment with A. U. was repeated. The *average* value for the two experiments was used in group calculations.

essentially devoid of citric acid, the citrate excreted in the urine, which must have been of "endogenous" or "metabolic" origin, increased in quantity with a shift of the body's acid-base equilibrium in the direction of alkalosis. Since consumption of grapefruit juice resulted in all cases in raising the urinary pH to a greater or less degree a correspondingly larger or smaller fraction of the increased citrate elimination is to be attributed to this circumstance. Therefore, although it is convenient to speak of the percentages in table 1 as that part of the citric acid of the grapefruit juice which the body "could not" ("failed to") oxidize, it should be remembered that as such they are somewhat too high.

With this reservation it may then be pointed out in summary that, of the citric acid contained in 1800 cc. of grapefruit juice, the sixteen men of the present study failed on the average to metabolize 1.76 ± 0.010%, while in the case of the nine women the fraction escaping oxidation averaged 2.27 ± 0.175%. Conversely, considering both sexes together, it thus appears that on the average at least 98% of the citric acid supplied in the grapefruit juice was utilized under the conditions of this experiment. In its availability to the body as a source of energy, citrate thus compares favorably with the better known and more abundant nutrients.

In terms of actual amounts of citric acid ingested and excreted, the men with an average intake of 22.49 gm. in the fruit juice excreted 0.399 ± 0.024 gm. "extra" in the urine; the women, with their intake increased by 21.29 gm., showed a mean rise in excretion of 0.468 ± 0.036 gm.

Despite the fact that the twenty-five subjects of these experiments were given from two to four times as much citric acid as those of Schuck, not one showed difficulty of efficient utilization even approaching that of her subject mentioned earlier, who excreted 1.294 gm. (16.6%) of the acid unchanged. The largest quantity of citric acid escaping oxidation in our series (case of M. C. U.) corresponds in actual amount to only three-fifths, and in percentage of the intake, to only one-fifth, of the comparable values for Schuck's "poorest" subject. The conclusion therefore seems warranted that cases of such

relatively poor utilization are seldom encountered, and that most persons are able to metabolize almost completely larger quantities of citric acid than they are apt to obtain through even very liberal consumption of citrus fruits.

Effect of grapefruit juice on urinary acidity. In each of the subjects observed, the pH of the 24-hour urine sample was higher on the day the juice was consumed than on the preceding control day. The average increase was 0.66 ± 0.044 pH unit for the entire group of twenty-five (0.67 for males; 0.64 for females).

Considering only the ten subjects whose diet (and hence their intake of acid- and base-forming elements) was the same, except for the grapefruit juice, on the control and experimental days, there appears a mean increase of 0.53 ± 0.11 unit over an average value of pH 6.00 on the control day. Further, excluding C. S. L. who, on the experimental day, took unaccustomed strenuous exercise and may for this reason have excreted extra lactic acid, the remaining nine best-controlled subjects show an increase in urinary pH of 0.58 ± 0.09, attributable to consumption of grapefruit juice.

It may possibly be argued that, though large intakes of citrus juice have a net alkalizing effect as shown in the 24-hour urine sample, there may, nevertheless, circulate for a short time sufficient extra acid citrate from the juice to produce a tendency toward increased acidity. However, the four short-time experiments briefly described above failed to yield evidence of such an effect, for no one of the successive samples of urine collected up to 22 hours after ingestion of the juice was more acid than the control sample collected the 2 hours before, and most of them were distinctly more alkaline.

The basal citric acid excretion

Effect of citrate intake. As might be expected from the ease with which the body was found to destroy almost completely the large amounts of citric acid in grapefruit juice, the variations in citric acid excretion among all individuals on low-citrate diets appear to bear little relation to the differences

in their citrate intakes. Thus, the Pearsonian coefficient, r, for the correlation between the intake and excretion of citric acid on the control day was $+ 0.086 \pm 0.198$ for the entire group; or, considering the sexes separately, $+ 0.156 \pm 0.325$ for the women and $+ 0.335 \pm 0.149$ for the men.

Even for the same individual, the increase of citric acid excretion when grapefruit juice is taken is not as dependent quantitatively on the increase of citric acid intake as might have been anticipated, r for this relationship being 0.410 ± 0.112 in the present series. Such a correlation coefficient, while probably "statistically significant", indicates that the two factors are associated only to a degree characterized as moderate by Chaddock ('25).

Relation to acid-base balance. In the experiments of Sherman, Mendel and Smith ('36 a) already cited, it was shown that for a given individual the excretion of citric acid tends to rise with an increase in urinary pH, and vice versa, whether the change in pH results from a shift in the articles of food consumed or through such means as the ingestion of sodium bicarbonate. In the present series, as pointed out above, the taking of large amounts of grapefruit juice in each case resulted both in an increase of citrate excretion and in a rise of urinary pH. That the extent of these changes is interdependent is clearly shown by the coefficient of $+ 0.640 \pm 0.118$ for the correlation between rise in urinary pH and rise in citrate excretion for a given individual. Indeed, the interesting observation that the degree of association is here marked, as contrasted with only a moderate positive correlation between citric acid intake and excretion (noted in the preceding paragraph) illustrates well the argument stressed earlier in this paper for regarding at least part of the increased citrate excretion as due to an acid-base shift per se.

However, as between different individuals, a consistent relation of urinary pH to basal citrate excretion was not found in the present series, r in the group of females being $- 0.334 \pm 0.296$, while for the males it was $+ 0.237 \pm 0.236$.

Sex difference. The basal excretion of citrate by the males averaged 0.403 gm. $\pm$ 0.021 while that of the females was

definitely higher, 0.643 ± 0.033 gm. Though we have no explanation for it, we are inclined to believe that this apparent sex difference is real.

SUMMARY

Twenty-five young adult subjects were found to utilize on the average 98% of the citric acid and citrates provided by very liberal (1800 cc. per day) consumption of grapefruit juice. Further evidences of a high tolerance toward citrus fruits were the absence of subjective symptoms and the finding in each case that consumption of grapefruit diminished the acidity of the 24-hour urine sample. Several additional studies in which a large (920 to 1675 cc.) single dose of grapefruit juice was taken and urine collections were made at short intervals showed a similarly high percentage utilization of the contained citric acid and a rise in urinary pH, i.e., a shift toward alkalinity, at all times up to 22 hours after ingestion of the juice.

We wish to express our thanks to Mrs. A. B. Rohrer, Mrs. Grace G. Stroup, and Dr. Oscar E. Lanford for their assistance with the analyses, and to the experimental subjects for their willing cooperation.

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THE DISTRIBUTION OF NICOTINIC ACID IN FOODS [1]

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The extensive use of nicotinic acid in the prevention and cure of pellagra makes it desirable to extend our knowledge about its distribution in foods. Information along this line has been rather meager because of the limitations of the methods of assay that have been employed. Bacharach ('41) has recently summarized most of the published data.

In general, the proposed microbiological methods for the determination of nicotinic acid have been limited in their application by relative inaccuracy, the necessity for clear extracts, and pathogenicity of the organisms. These difficulties have been largely eliminated in the microbiological method of Snell and Wright ('41) which has proven to be very satisfactory in our hands.

EXPERIMENTAL

The method is based on the response of Lactobacillus arabinosus 17-5 to nicotinic acid or to its amide. Acid production is plotted against known nicotinic acid concentration to obtain a standard curve. The nicotinic acid content of the unknown is obtained by interpolation on the standard curve. Acid production is proportional to nicotinic acid concentration from 0.04 to 0.20 μg. of nicotinic acid per tube.

A number of workers, in attempting to use the method of Snell and Wright, have experienced difficulty in obtaining low blanks. The assay of medium constituents has shown that casein, biotin concentrates, and cystine preparations may

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be sources of nicotinic acid contamination.[2] Labco casein or acid-washed casein prepared according to the method of Arnold and Elvehjem ('38) give satisfactory blanks. The amount of casein used in the medium contributes from 0.7 cc. to 1.0 cc. to the blank titration. Extraction of the casein with alcohol reduces this amount to 0.5 cc.

The original procedure recommends incubation at 30°C. It has been found that incubation at 37°C. gives identical results.

TABLE 1

Comparison of various methods of extraction in the microbiological determination of nicotinic acid.

SAMPLE	METHOD OF EXTRACTION		
	H_2O	HCl	NaOH
	mg.%	*mg.%*	*mg.%*
Skim milk powder I	0.88	0.90	0.89
Liver concentrate 14600	114	112	112
Whole wheat I	4.9	6.9	6.8
Whole wheat II	4.5	6.1	6.1
Whole wheat III	4.0	5.1	5.3
Patent flour I	0.90	1.15	1.18
Patent flour II	1.07	......	1.05
Patent flour III	1.10	......	1.30
Dried baker's yeast I	40.0	......	41.2
Dried grass concentrate	16.0	......	15.5
Dried cows' rumen contents	6.3	......	6.2
Dried fried ham 141	......	18.0	19.5
Dried beef liver 98	......	43.0	42.3
Dried beef spleen 76	......	18.5	19.7

Preparation of sample

The stability of nicotinic acid makes it possible to use rather severe treatments in order to obtain good extraction. The effectiveness of various methods of extraction is shown in table 1. For most materials, water extraction is satisfactory. However, in the case of cereals, alkaline or acid treatment is necessary for complete extraction. Water extraction was carried out by suspending from 0.1 to 0.5 gm. of finely ground

[2] Cystine hydrochloride obtained from the Eastman Kodak Co., Rochester, New York, and biotin concentrates from the S.M.A. Corp., Chagrin Falls, Ohio, have been found to be satisfactory. At present crystalline biotin is available and should, of course, be used in preference to impure biotin concentrates.

material in 75 cc. of water, autoclaving for 20 minutes at 15 pounds pressure, and diluting to the desired volume. Treatment with HCl involved heating from 0.1 gm. to 0.5 gm. of material on a steam bath for 1 hour with 25 cc. of 5 N HCl, neutralizing with NaOH (brom thymol blue is used as an outside indicator), and diluting to a convenient volume. The NaOH treatment was carried out as follows: From 0.1 gm. to 0.5 gm. of finely ground material is suspended in approximately 75 cc. of water, 20 cc. of 8% NaOH are added, and the suspension is autoclaved at 15 pounds pressure for 20 minutes. It is then neutralized with HCl and diluted to a convenient volume.

TABLE 2

Recovery of added nicotinic acid.

SAMPLE	NICOTINIC ACID FOUND IN ORIGINAL SAMPLE	NICOTINIC ACID ADDED	TOTAL NICOTINIC ACID FOUND	PER CENT RECOVERY
	µg./gm.	*µg./gm.*	*µg./gm.*	
Whole wheat	49	50	100	102
Dried baker's yeast I	400	500	880	96
Patent flour IV	12	25	38	104
Yellow corn	24	50	75	102
Beef kidney 126	315	250	560	98
Evaporated milk I	1.8	5	7.2	108
Beef liver 98	455	500	904	90

If a sample of whole wheat is being assayed, a 0.2 gm. sample containing approximately 12 µg. of nicotinic acid is diluted to a final volume of 250 cc. Each cubic centimeter of solution then contains approximately 0.05 µg. of nicotinic acid and aliquots from 1 cc. to 4 cc. fall in the range of the assay.

In these studies, fresh fruits and vegetables were homogenized in a Waring Blendor, and a water suspension was then treated with NaOH as outlined above.

Recovery of nicotinic acid when added to a wide variety of materials ranged from 90% to 110%, as shown in table 2. These results substantiate the conclusion of Snell and Wright ('41) that ordinary materials do not contain interfering amounts of inhibitory or stimulatory substances.

In order to study the assay of food mixtures, various combinations of liver, corn, dried grass, yeast, rice, skim milk powder, wheat and bananas have been assayed. As shown in table 3, the values obtained checked the calculated values based on the assay of each component.

TABLE 3

The assay of food mixtures for nicotinic acid.

MIXTURE I	NICOTINIC ACID	MIXTURE III	NICOTINIC ACID
	$\mu g.$		$\mu g.$
Banana 0.7 gm.	4.5	Whole wheat 0.5 gm.	30.5
Pork liver 0.1 gm.	54.0	Skim milk powder 0.5 gm.	5.0
Corn meal 1.0 gm.	10.3	Dried grass 0.5 gm.	36.0
Skim milk powder 0.5 gm.	5.0		71.5
	73.8	*Found*	75.0
Found	72.0		
MIXTURE II		MIXTURE IV	
Baker's yeast 0.1 gm.	40.0	Pork liver 0.1 gm.	54.0
Brown rice 0.5 gm.	34.5	Corn meal 1.0 gm.	10.3
Whole wheat 0.5 gm.	30.5	Dried grass 0.5 gm.	36.0
Dried grass 0.5 gm.	36.0	Yeast 0.1 gm.	40.0
	141.0	Brown rice 0.5 gm.	34.5
Found	150.0	Skim milk powder 0.5 gm.	5.0
		Whole wheat 0.5 gm.	30.5
		Banana 0.7 gm.	4.5
			214.8
		Found	217.0

TABLE 4

Comparison of chemical and microbiological assays of dried meats for nicotinic acid.

SAMPLE	CHEMICAL ASSAY	MICROBIOLOGICAL ASSAY
	mg.%	*mg.%*
Pork kidney 62	47.9	45.5
Pork loin 74	21.4	23.5
Pork ham 95	27.0	28.0
Fried beef liver 121	53.3	38.0
Veal liver 70	50.8	41.0
Veal hindquarter 134	31.4	29.0
Beef pancreas 64	18.0	15.7
Light chicken 68	29.7	29.0

TABLE 5

Nicotinic acid content of fruits, vegetables, cereals and miscellaneous materials.

FRUITS AND VEGETABLES

Sample	Fresh	Dry
	mg.%	*mg.%*
Apple (peeled)	0.50	3.54
Apple peelings	1.13	7.15
Banana	0.61	2.90
Beans, wax	0.76	7.45
Beans, green	0.64	6.09
Beans, kidney		2.82
Beans, lima		1.83
Cabbage	0.29	4.46
Carrot	1.47	14.0
Cauliflower	0.57	7.13
Cranberries	0.13	1.23
Cucumber	0.32	8.0
Dates	2.18	
Grapes (Thompson seedless)	0.28	1.84
Kohlrabi	0.27	3.86
Onion	0.10	0.77
Peach (peeled)	0.95	7.92
Pear (peeled)	0.14	1.15
Pear peelings	0.34	1.42
Potato, white (peeled)	1.18	5.54
Potato, white, peelings	1.01	5.05
Potato, sweet (peeled)	1.29	4.53
Potato, sweet (peelings)	1.71	4.97
Plum (peeled)	0.56	3.86
Plum peelings	0.67	3.52
Raisins	0.63	
Red beet	0.64	4.74
Soybean flour		2.40
Spinach	0.72	7.65
Tomato (peeled)	0.58	16.5
Tomato peelings	0.76	9.50
Yam (peeled)	0.67	2.20
Yam peelings	1.51	4.80
	mg. per 100 cc.	
Grapefruit juice	0.21	
Lemon juice	0.08	
Lime juice	0.09	
Orange juice	0.22	
Tomato juice	0.10	

CEREALS

Sample	
	mg.%
Oats — Sample I	1.13
Oats — Sample II	1.60
Red barley	4.70
Pearled barley	2.75
Buckwheat	4.40
Polished rice	0.90
Brown rice	6.90
Rice polishings	96.6
Rye — Sample I	0.90
Rye — Sample II	1.03
Rye — Sample III	1.29
Yellow corn (8 samples)	1.56–2.60
Yellow corn meal — Sample I	1.03
Yellow corn meal — Sample II	1.08
White corn meal	1.76
Whole wheat	5.0–7.0
Milling fractions of wheat	
Patent flour	0.80
Fancy first clear flour	1.68
Second clear flour	5.55
Clean shorts	8.44
Wheat feed and screenings	19.2
Bran — Sample I	25.7
Bran — Sample II	28.7
Bran — Sample III	40.0
Seminola	2.01
Farina	0.98
Standard middlings and screenings	13.0
Flour middlings and screenings	9.2
Durum middlings	17.7
Wheat middlings — Sample II	10.6
Wheat germ — Sample I	3.4
Wheat germ — Sample II	4.7
Defatted wheat germ	6.6
Wheat gluten	2.5

MISCELLANEOUS MATERIALS

Sample	
	mg.%
Corn silage	1.5
Linseed oil meal	4.3
Timothy hay	2.3
Alfalfa meal	3.9
Malt sprouts	5.6
Dried grass	7.2
Whole milk	0.08
Evaporated milk I	0.18
Acidophilus milk	0.08
Skim milk powder	0.89
Human milk	0.26
Macaroni	2.10
Peanut butter	18.6
Egg white (hard boiled)	0.076
Egg yolk (hard boiled)	0.035
Paprika	1.38
Canned shrimp	0.78
Canned oyster	0.66
Canned clam	1.08
Brewer's yeast	40.0–60.0
Baker's yeast	40.0–50.0
Dried blood	2.7
Breads	*Fresh mg.%*
White bread I	0.68
White bread II	0.95
White bread III	0.66
Enriched white bread	1.51
Milk bread	0.92
Whole wheat blend I	1.80
Whole wheat blend II	1.70
100% whole wheat bread	2.88
Potato bread	0.81
Soybean bread	1.35

DISCUSSION

In general, values obtained by microbiological assay agree quite well with values obtained by chemical analysis. In the assay of meats, excellent agreement has been obtained, as shown in table 4. The values for the chemical assay are taken from the report of McIntire, Waisman, Henderson and Elvehjem ('41). The values for kidney, muscle and pancreas agree very well, the only marked discrepancy being in the case of liver. The microbiological assay of liver gives results that are somewhat lower.

Most fruits and vegetables are not very good sources of nicotinic acid (table 5). On a dry weight basis, carrots and tomatoes are the best of this group. Meats in general, particularly the organs, yeast and peanut butter are excellent sources. Milk is very low in nicotinic acid content, containing about 0.08 mg. per cent. Eggs are also a very poor source.

TABLE 6

The nicotinic acid content of soft wheat flours obtained from various sections of the United States.[1]

STATE FROM WHICH SAMPLE WAS OBTAINED	PATENT FLOUR	CLEAR FLOUR	CUT OFF FLOUR
	mg.%	*mg.%*	*mg.%*
Michigan	0.80	1.11	
Tennessee	0.79	1.00	
Missouri	0.82	1.18	
North Carolina	0.76	1.02	
Kentucky	0.75		1.43
Tennessee	0.84		1.30

[1] The authors wish to express their thanks to Mr. W. H. Strowd, National Soft Wheat Millers Association, Nashville, Tennessee, for providing the flour samples listed in this table.

Table 5 shows that whole cereals vary considerably in their nicotinic acid content. Corn, oats, and rye are all quite low; buckwheat, barley, and whole wheat contain from 4.5 to 7.0 mg. per cent; white flour contains about 1.0 mg. per cent.

The analysis of various milling fractions of wheat (table 5) indicates that most of the vitamin is contained in the bran and middling fraction. Patent flour is low, but some nicotinic acid is retained in the coarser clear and cutoff flours (table 6).

SUMMARY

A study has been made of the application of the microbiological method of Snell and Wright ('41) to the assay of foods for nicotinic acid. The nicotinic acid content of a variety of foods is reported.

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THE EFFECT OF AROMATIC HYDROCARBONS ON THE GROWTH OF YOUNG RATS

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ONE CHART

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In a preliminary communication (West, '40) it was reported that complete cessation of growth is caused by the introduction of 1% diphenyl into a 6% casein diet fed to young rats. The condition is relieved and growth is resumed when either l-cystine or dl-methionine is added to the diet. It was supposed that the hydrocarbon is detoxicated through a mechanism involving the sulfur-containing amino acids.

In view of the fact that the decreased food consumption observed in the experimental animals might conceivably be interpreted as causing the lowered growth rate, and further, since the improvement in food consumption and growth following addition of the sulfur-containing amino acids to the hydrocarbon diet might be attributed to the effect of these acids in increasing the nutritive value of a casein diet, it was thought that experiments designed to rule out these factors as being responsible for the results previously observed should be undertaken. These as well as similar experiments with chrysene are here presented.

EXPERIMENTAL

The basal diet contained in per cent: casein, 6; salt mixture (Osborne and Mendel, '19), 4; sucrose, 15; cod liver oil, 1; hydrogenated cottonseed oil[1], 24; and corn starch, 50. The

[1] Crisco.

quantity of starch added to the supplemented diets was reduced by amounts equivalent to the weight of the material added. Experiments were carried out with supplements of either (a) hydrocarbons alone (0.3% diphenyl; 0.3% chrysene), (b) amino acids alone (0.120% l-cystine; 0.150% dl-methionine), or (c) both hydrocarbons and amino acids [2].

Young white rats from our stock colony were placed on each of the diets, respectively, shortly after weaning. They were kept in raised-bottom cages and allowed water and food ad libitum. The experiments lasted 30 days. Each rat received daily 0.4 gm. of brewers' yeast [3].

In a second series of experiments paired-feeding tests were carried out with litter mates on different diets.

RESULTS

The results of the ad libitum experiments are shown in chart 1. Both diphenyl and chrysene retarded the growth rate in the amounts given, chrysene being somewhat more active in this respect. Addition of either l-cystine or dl-methionine, to the basal diet increased both the rate of growth and the food consumption. Diets containing both hydrocarbon and amino acid gave intermediate values.

The paired-feeding experiments (table 1) eliminated the differences in food consumption but showed the same definite trend observed in growth rates in the ad libitum experiments.

DISCUSSION

The results of the experiments demonstrate that depression of growth by the hydrocarbons is not due to the decreased food consumption. The hydrocarbons evidently react in some manner with the sulfur-containing amino acids since growth rates

[2] The l-cystine was C. P. Cystine purchased from the Coleman and Bell Company. It was recrystallized and analyzed. The dl-methionine was purchased from the Research Laboratory, Eastman Kodak Company. The diphenyl was a pure sample melting at 69° C. The chrysene was kindly furnished us by Dr. N. O. Calloway. It had been recrystallized ten times and melted at 247° C. (Melting points are uncorrected.)

[3] Mead's.

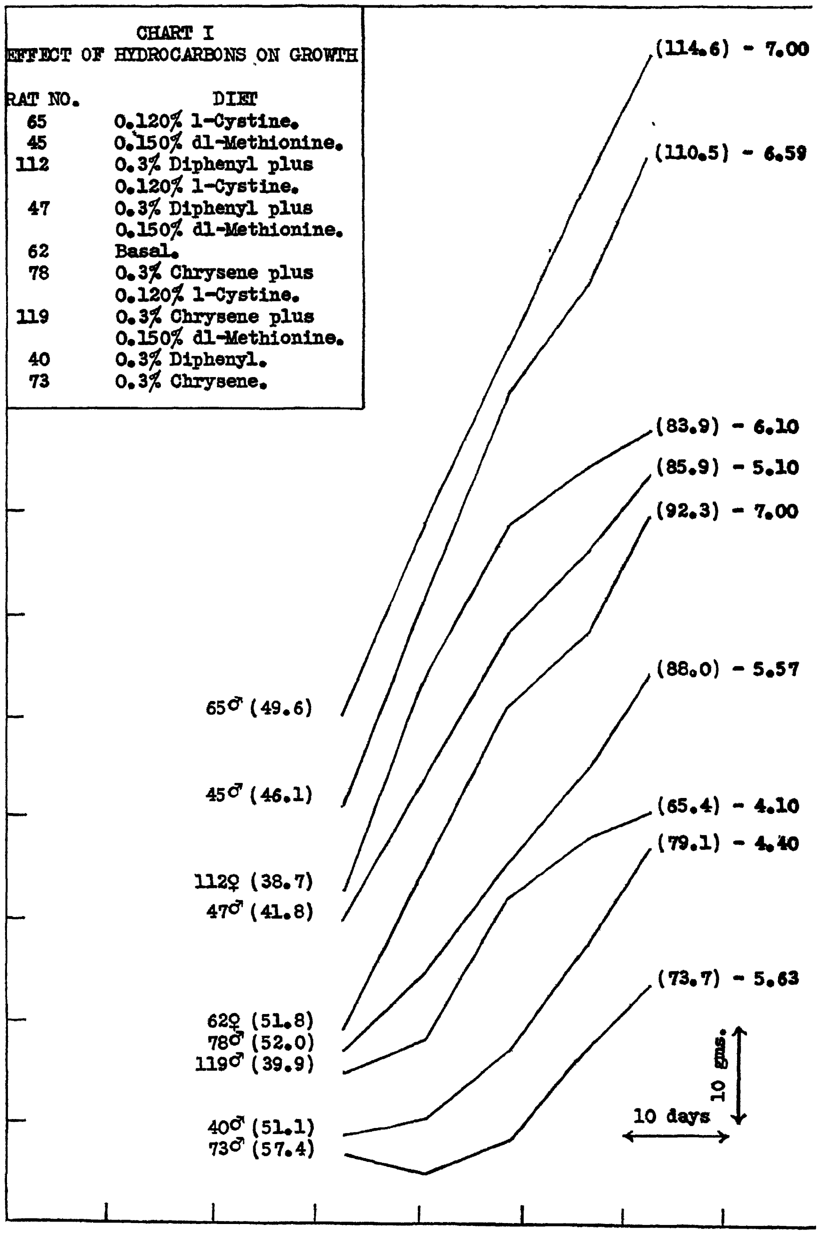

Chart 1 Initial and final weights are given in parentheses. The average daily food consumption in grams is placed after the final weight. Each curve is typical of results with at least three to five animals.

intermediate between those observed for the hydrocarbon supplements alone and for the amino acid supplements alone were obtained when the aromatic compounds were added to the same diet. Another argument which points toward the fundamental nature of the phenomenon is based on the fact that varying the amount of hydrocarbon gave results which varied directly with the quantity fed. When the basal diet

TABLE 1

Growth of young rats for 30 days on the basal diet with and without supplements. Paired-feeding experiments.

DIET	RAT NO.	SEX	BODY WEIGHT			DAILY FOOD INTAKE
			Initial	Final	Daily change	
Basal	61	♀	55.6	90.3	1.15	3.80
0.3% Diphenyl	60	♀	50.3	76.1	0.83	3.80
Basal	86	♀	59.3	107.5	1.60	6.37
0.3% Diphenyl	85	♀	71.6	99.6	0.93	6.37
Basal	98	♂	41.3	80.6	1.26	6.51
0.120% l-Cystine	99	♂	46.4	100.0	1.73	6.51
Basal	100	♀	40.3	81.2	1.31	6.43
0.120% l-Cystine	101	♀	40.7	94.7	1.74	6.43
Basal	102	♂	67.9	102.0	1.16	7.03
0.150% dl-Methionine	103	♂	69.9	121.0	1.73	7.03
Basal	104	♂	66.7	103.7	1.23	6.93
0.150% dl-Methionine	105	♀	61.2	117.1	1.86	6.93
Basal	107	♀	64.2	89.2	0.86	5.49
0.3% Chrysene	106	♀	61.0	76.4	0.51	5.49
Basal	109	♀	60.2	86.2	0.86	5.08
0.3% Chrysene	108	♀	63.7	75.1	0.38	5.08

was supplemented with 0.2% diphenyl the average daily change in weight for two animals in 30 days was 0.99 gm.; with 0.3%, 0.63 gm.; with 0.4%, 0.55 gm.; and with 0.5%, 0.39 gm. Supplements of 0.2% and 0.5% chrysene allowed a daily average increase in weight of 0.79 gm. and 0.52 gm., respectively.

The experimental results indicate that chrysene is somewhat more active than diphenyl in depressing the growth of

the animals. The reason for this difference is not apparent at the present time. Chrysene may be more toxic than diphenyl. Also, since chrysene is known to be very mildly carcinogenic (Cook, Haslewood, Hewett, Hieger, Kennaway and Mayneord, '27) this may be a factor (Haddow, Scott and Scott, '37), but sufficient proof of the contention that carcinogenic compounds are more active than non-carcinogenic compounds in retarding the growth of rats on a low protein diet is still lacking (White and White, '39).

Attempts in this laboratory to demonstrate the presence of a sulfur-containing detoxication product in the urine of rats fed diphenyl and chrysene have so far met with failure. However, some evidence has been adduced for the fact that one detoxication product of diphenyl is a crystalline phenol. These results will be the subject of a subsequent report. Of interest in this connection is the finding of Stroud ('40) that a yield of 25.4% of 4-hydroxydiphenyl can be achieved in isolation experiments using the urine of rabbits fed diphenyl.

While the growth experiments clearly indicate that the sulfur-containing amino acids are involved in the metabolism of the hydrocarbons it does not seem mandatory that the final detoxication product should contain sulfur; nor does the proof of the mediation of these amino acids in the detoxication process call for the isolation of a mercapturic acid or similar derivatives since it has been shown that synthetic sulfhydryl and cysteine derivatives of certain carcinogenic compounds are quite unstable, being easily convertible even in neutral aqueous medium to the corresponding disulfides (Wood and Fieser, '40). The products could then conceivably yield the corresponding carboxyl or hydroxyl derivatives (Stekol, '40).

CONCLUSIONS

1. Both diphenyl and chrysene retard the growth of young rats on a low protein diet.

2. This condition is relieved by introduction of either l-cystine or dl-methionine into the hydrocarbon diets.

3. By employing the method of paired-feeding it has been shown that the depression of growth of the animals on the hydrocarbon diets is not due to the lowered food consumption observed in the ad libitum experiments.

4. The paired-feeding experiments also strengthen the conclusion that the alleviation of the condition imposed by the hydrocarbons is not due to the effect of the sulfur-containing amino acids in increasing the nutritive value of the basal diet but to the increased demands of the tissues for these acids under the experimental conditions.

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THE FATE OF EXCESS VITAMIN A STORES DURING DEPLETION

VALUE OF THE HISTOLOGIC DEMONSTRATION OF VITAMIN A [1]

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ONE PLATE (EIGHT FIGURES)

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Studies on the histologic demonstration of vitamin A in rat organs by means of fluorescence microscopy (Querner, '35; Popper, '40) have shown that high values for vitamin A fluorescence are seen in the livers of animals that have received large amounts of the vitamin (Popper and Greenberg, '41). The fluorescence is chiefly visible in the endothelial lining of the blood sinusoids of the liver, in the Kupffer cells, and to only a slight extent in the epithelial cells. Davies and Moore ('35) reported that when excessive amounts of vitamin A were fed, a rat liver could store enough of the vitamin to maintain the animal for a century. If such a rat was kept on a vitamin A deficient diet this enormous amount of vitamin A was quickly lost. In a later publication, Moore ('40) related this rapid drop in the initial depletion time of such rats to a lack of vitamin E. This non-economic utilization of vitamin A when given in huge amounts could be explained by the histologic picture: the Kupffer cells store the excess of vitamin A and the liver cells constitute the only permanent storage place in the body. The excess seen in the Kupffer

[1] This work was supported by a grant from the Committee on Scientific Research of the American Medical Association.

cells may either be destroyed there or given up to other organs by way of the blood stream.

To study these possibilities we have repeated the experiments of Davies and Moore. In addition to the chemical assay of blood and livers for vitamin A the changes in the histologic distribution were observed in rats first given large amounts of vitamin A and then placed on a vitamin A deficient diet. It was hoped to throw some light on the role of the Kupffer cells both in hypervitaminosis and during deficiency since the importance of these cells in vitamin A metabolism is indicated (Uotila and Simola, '38; Hirt and Wimmer, '40; Popper and Greenberg, '41).

In this study chemical and histologic examinations of the liver with various gradations of the vitamin A content were made; this gave us a chance to evaluate the histologic method for demonstration of vitamin A.

MATERIAL AND METHODS

Out of ten litters, fifty-three rats were used which were as close to 4 weeks of age as possible with a maximum age variation of 6 days. Their mothers had been fed Sherman's diet 13. These animals were given large amounts of vitamin A prior to depletion by supplementing diet 13 with a vitamin A concentrate administered orally. During the first week each rat received 20,000 I.U. of vitamin A [2] daily. This dosage was discontinued due to the development of the toxic signs of hypervitaminosis A (Drigalski, '33; Domagk and Dobeneck, '33); after 3 days 10,000 to 12,000 I.U. of vitamin A [3] were administered daily for 28 days. Two male and two female rats were then sacrificed. The livers were examined histologically and chemically for the vitamin A content; the eyes were examined histologically and the pooled blood serum chemically for the vitamin A content. The remaining rats were placed on a vitamin A free diet and at suitable intervals groups of four animals (two male and two female) nearly alike

[2] Shark liver oil concentrates of Vitamin Inc., Chicago, containing approximately 1,000,000 I.U. of vitamin A and 3333 I.U. of vitamin D in 1 gm., were used after dilution with cottonseed oil.

[3] Halibut liver oil contributed by Mead Johnson Company.

in weight and age were sacrificed and the analyses made.[4] Some of the eyes were examined after removal under dark red light following 12 hours of dark adaptation; others after light adaptation.

The chemical method used for determination of vitamin A was that of McCoord and Luce-Clausen ('34), using the Evelyn Photoelectric Colorimeter. The values were recorded as Evelyn Photoelectric Colorimeter Units.[5]

Parts of the livers and the eyes were fixed for 12 hours in formaldehyde solution (U.S.P.). Frozen sections were mounted in water and then examined under the fluorescence microscope. The details of the technic are described elsewhere (Popper, '41). Vitamin A imparts a bright green fluorescence which is quickly destroyed by ultra violet light. This fading fluorescence is imparted by lipoids in tissue sections in a degree parallel to the amount of vitamin A present. A mercury vapor bulb, rich in ultra violet rays, serves as the light source. A filter system absorbs the visible light. A standard microscope is used. Fluorescence tissue constituents change the ultra violet light to various colors of visible light. The remaining ultra violet rays are absorbed by a filter over the eye piece. Since many tissue constituents impart some kind of fluorescence, the orientation is almost as easy in an unstained section under ultra violet light as it is in a stained section in visible light. The vitamin A fluorescence is differentiated from other kinds of fluorescence by the green color, the brightness and the fading. In addition, frozen sections stained with methylene blue and with phosphin 3R, a sensitive fat stain, were examined. In the first an exact localization of the fluorescence is possible if the ultra violet filter is changed to a ground glass filter. Photomicrographs were taken with a small film camera (Popper and Elsasser, '41).

[4] When one rat could not provide enough serum, the blood of two in the group was pooled. The last two groups consisted of two and three rats, respectively.

[5] The liver and blood values refer to Evelyn Photoelectric Colorimeter units per 100 gm. liver and per 100 cc. of serum, respectively. We found an average of 235 units per 100 gm. liver and 10.2 units per 100 cc. of serum in adult stock animals on diet 13 (Brenner et al., '42).

RESULTS

The results obtained are shown in table 1 in which the total amount of fluorescence in the liver is indicated by crosses. Even with a depletion period of 30 weeks no complete exhaustion of the vitamin A depots was found with the chemical and histologic method. Through the entire range from "hypervitaminosis" down to almost avitaminosis the chemical assay corresponded with the amount of fluorescence. In the lower ranges the parallelism between histologic and chemical methods was more striking than was indicated by the rough way of marking with crosses. In the highest ranges (above 7000 units) the histologic picture only revealed large amounts of vitamin A without definite differences.

The variations of the histologic picture were characteristic. In the hypervitaminotic stage the Kupffer cells were extremely rich in the fluorescence and irregular in shape (fig. 1). The fading required several minutes and after that a dim bluish white fluorescence of the Kupffer cells remained indicating a high fat content. The liver cells revealed a moderate vitamin A fluorescence localized in fine lipoid droplets which lined the edge of the cells like pearls on a string. Their cytoplasm imparted a diffuse, much dimmer, green fading fluorescence. The Kupffer cell fluorescence exceeded by far that of the liver cells. In the periportal fields and the areas around the central veins fine droplets with vitamin A fluorescence were seen, apparently in endothelial and other connective tissue cells. The vitamin A fluorescence was homogenous throughout the lobules.

Starting from the second week the total amount of fluorescence diminished, due to a decrease in the Kupffer cell fluorescence. These cells appeared less irregular and their fluorescence faded more quickly and completely (fig. 2). When the chemical value dropped below 4000 to 5000 units the prevalence of the fluorescence in the Kupffer cells disappeared. The liver cell fluorescence remained unchanged; one even gained the impression that it increased, although this may have been an optical illusion. Somewhat later the

TABLE 1

Comparison of the vitamin A content of blood and liver (determined chemically) with the vitamin A fluorescence of the liver in hypervitaminotic rats on a vitamin A deficient diet.

TIME OF DEPLETION	SEX	VITAMIN A FLUORESCENCE [1] Total amount	Liver cells	Kupffer cells	Localization	CHEMICAL ASSAY FOR VITAMIN A Liver	Blood serum
						Units/ 100 gm.	*Units/ 100 cc.*
0	F.	+++++	+++	+++++	Diffuse	12,931	40
0	F.	+++++	+++	+++++	Diffuse	11,748	
0	M.	+++++	+++	+++++	Diffuse	10,768	
0	M.	+++++	+++	+++++	Diffuse	7,498	
3 days	F.	+++++	+++	+++++	Diffuse	14,628	
3 days	M.	+++++	+++	+++++	Diffuse	7,661	16.9
3 days	F.	+++++	+++	+++++	Diffuse	16,674	
3 days	M.	++++	+++	+++++	Diffuse	7,714	20.4
1 week	F.	+++++	+++	+++++	Diffuse	19,659	
1 week	M.	+++++	+++	+++++	Diffuse	14,420	20.2
1 week	F.	+++++	+++	+++++	Diffuse	14,533	
1 week	M.	++++	+++	++++	Diffuse	11,735	17.2
2 weeks	F.	++++	+++	++++	Diffuse	5,733	
2 weeks	F.	++++	+++	++++	Diffuse	6,473	11.5
2 weeks	M.	+++	+++	+++	Diffuse	5,525	14.0
2 weeks	M.	++	+++	++	Diffuse	1,801	12.4
3 weeks	F.	++++	+++	++++	Diffuse	4,544	
3 weeks	F.	++++	+++	++++	Diffuse	4,293	9.4
3 weeks	M.	++++	+++	+++	Diffuse	4,413	
3 weeks	M.	++++	+++	+++	Diffuse	5,762	19.8
4 weeks	F.	++++	+++	+++	Diffuse	4,938	
4 weeks	F.	+++	+++	++	Diffuse	2,562	8.8
4 weeks	M.	++	++	++	Diffuse	1,570	
4 weeks	M.	++	+++	++	Diffuse	1,767	14.7
5 weeks	F.	++++	+++	+++	Diffuse	2,968	
5 weeks	F.	++++	+++	++++	Diffuse	4,597	13.1
5 weeks	M.	++	++	++	Central	759	14.9
5 weeks	M.	+++	+++	++	Diffuse	2,919	19.0
6 weeks	F.	+++	+++	++	Diffuse	1,948	
6 weeks	F.	++++	+++	++++	Diffuse	4,547	7.7
6 weeks	M.	++	++	++	Peripheral	717	10.8
6 weeks	M.	++	++	++	Peripheral	980	16.3
8 weeks	F.	+++	+++	+++	Diffuse	1,816	
8 weeks	F.	+++	+++	+++	Diffuse	1,950	8.8
8 weeks	M.	++	++	+	Diffuse	694	14.9
8 weeks	M.	++	++	+	Diffuse	674	9.8
10 weeks	F.	++	++	+	Diffuse	965	
10 weeks	F.	+++	++	++	Diffuse	1,265	8.6
10 weeks	M.	+	+	++	Peripheral	238	16.1
10 weeks	M.	++	++	++	Peripheral	350	14.7
13 weeks	F.	++	++	+	Diffuse	863	
13 weeks	F.	+++	++	++	Diffuse	2,458	6.5
13 weeks	M.	+	±	+	Central	228	
13 weeks	M.	+	±	+	Central	112	10.5
18 weeks	F.	++	++	++	Diffuse	501	
18 weeks	F.	++	++	++	Central	364	6.6
18 weeks	M.	±	+——	±	Central	71	9.6
18 weeks	M.	++	++	±	Diffuse	274	7.3
23 weeks	F.	+	±	+	Diffuse	211	6.5
23 weeks	M.	±	—	±	Central	80	8.7
30 weeks	F.	++	++	+	Diffuse	449	8.8
30 weeks	F.	+	+	±	Central	173	8.6
30 weeks	M.	±——	—	±——	Central	50	8.4

[1] The vitamin A fluorescence of an adult stock animal on diet 13 corresponds to 2 plus signs.

fluorescent droplets in the neighborhood of the vessels disappeared.

Livers containing from 700 to 4000 units revealed the picture of a normal liver rich in vitamin A in a homogenous distribution; the fluorescence of the liver cells was parallel or even greater than that of the Kupffer cells (figs. 3, 4) although it gradually diminished with advancing depletion. When the total amount dropped below 700 units the continuous row of fluorescent droplets on the edge of the liver cells disappeared and the droplets were only found in the vicinity of the Kupffer cells (figs. 5, 7). Again the Kupffer cells showed a preponderance of fluorescence which became definite when the total amount fell below 250 units (fig. 6). The homogenous distribution disappeared; some part of the lobules, usually the periphery, was first depleted. Gradually the dim fluorescence of the cytoplasm of the liver cells disappeared. The last remnants of the vitamin A fluorescence were imparted by a few Kupffer cells in the central part of the lobules (fig. 8). In the periportal fields some ultra violet, stable brown or yellow inclusions became visible. The fat distribution followed that of vitamin A rather closely.

In the parallel study of Brenner, Brookes and Roberts ('42) based on the chemical assay a definite sex difference has been established. The amount of vitamin A in the liver after controlled feeding is higher in female rats and during depletion the male rats lose vitamin A much faster than the females. The first point was not substantiated histologically because no reliable differences were recognized in the higher ranges; the greater speed of depletion in males was histologically quite apparent. All the stages described above were reached faster by the male than by the female rats. In the males the preponderance of the fluorescence of the Kupffer cells disappeared and reappeared more rapidly than in females, in accordance with the faster drop of the total amount (compare fig. 4 with fig. 5, and fig. 6 with fig. 7). Complete lack of vitamin A fluorescence was reached neither in the males nor

females, but the fluorescence in the males at the end of 30 weeks was much lower than was that of the females.

The serum vitamin A level did not show any relation to the total amount of liver fluorescence or the fluorescence of the Kupffer or liver cells. In the males in which the Kupffer cell fluorescence was lost more quickly than in the females the blood level was higher, but occasionally a high blood level was associated with high Kupffer cell fluorescence in either sex. In the lower blood ranges especially, various degrees of Kupffer cell fluorescence were encountered, indicating the independence of the blood level from the Kupffer cell vitamin A.

The results of the eye studies agreed with the findings of Greenberg and Popper ('41). In the retina of dark adapted hypervitaminotic rats no vitamin A fluorescence was found. In light adapted eyes the fluorescence was found in high degree in the pigment coat, and in lesser degree in the rod and cone layer. In the former it was seen imparted by fine granules lining the hexagon shaped cells. Independent of the state of light adaptation the fluorescence was seen in the connective tissue cells of the ciliary processes in animals only after short depletion, but disappeared when the vitamin A in the liver dropped below 6000 units. The fluorescence of the retina did not entirely disappear, but seemed to diminish somewhat after a depletion period of 18 weeks.

DISCUSSION

The histologic and chemical methods agree in the estimation of the relative amounts of vitamin A present in the liver, except in the highest ranges where quantitative differences in the excess of fluorescence are not recognized histologically. This agreement is an additional evidence for the value and specificity of the histologic method. Furthermore, it permits the substitution of a much simpler histologic method for the chemical assay if a rough estimation of the vitamin A content of the liver is desired; in addition information on the localization of vitamin A is obtained. The histologic method is far superior if only parts of the structures of an organ

contain vitamin A. The chemical assay may not demonstrate this when these structures are buried in the great mass of vitamin A free tissue.

The importance of the localization is shown by the histologic studies. In the phase of high vitamin content there is a characteristic preponderance of the vitamin A storage in the Kupffer cells. In the normal stage relatively more is found in the liver cells than in the Kupffer cells. Whether an increase in the liver cells actually occurs, is not clear from the histologic picture. A decrease of the liver cell fluorescence, however, in the early stage of depletion of the animals which have received large amounts of the vitamin can be excluded. Our studies confirm the rapid loss of the vitamin A depots in the first 4 weeks (Davies and Moore, '35) with the chemical method, and less clearly with the histologic method, when the total amount of fluorescence is considered; however, the decrease found by us is somewhat slower. The histologic picture indicates that this rapid loss of the vitamin A stores of the liver concerns only the depots of the Kupffer cells. There is no relation between the blood levels at this time and the Kupffer cell fluorescence. Thus, there is no evidence that the Kupffer cells discharge the excess of vitamin A into the blood and we may assume that the Kupffer cells destroy this excess, thereby explaining the rapid loss.

Moore connected this non-economic utilization with a vitamin E deficiency of the rats since vitamin E seems to influence vitamin A storage in the liver (Moore, '40; Bacharach, '40). The possibility that vitamin E or its absence may be related to the destruction of vitamin A in the Kupffer cells could be considered. In our rats, however, there is no evidence for a vitamin E deficiency from the food intake, at least not in the early stages of depletion.

In the long middle period of depletion when a normal vitamin A store is present, there is preponderance of the fluorescence in the liver cells. Gradually their fluorescence decreases; this points to the liver cells as the physiologic stores for vitamin A. In advanced depletion a preponderance of

the Kupffer cell fluorescence reappears. The remnants of the vitamin A fluorescence in the liver cells are imparted by a few lipoid droplets adjacent to the Kupffer cells; previously, the entire edge of the liver cells had been lined by such droplets. Finally, only the Kupffer cells reveal the fluorescence. Apparently the Kupffer cells are concerned with the transmission of the vitamin A from the liver cells to the blood.

The lack of parallelism between the blood vitamin A level and the vitamin A fluorescence of the liver (which holds also for the amount of vitamin A chemically found in the liver), at least under the conditions studied, should be stressed. It coincides with the chemical analyses of Brenner, Brookes and Roberts ('42) who reported in greater detail the relation of the blood level to the liver stores and discussed the significance of their values. Blood levels of somewhat below 10 units are found in rats whose liver vitamin A fluorescence varies from $+++$ to the nearly deficient $\pm --$.

As is already known (Greenberg and Popper, '41), the vitamin A fluorescence of the rat's retina depends much more on the state of light adaptation than on the nutritional state of the animal. No relation between the vitamin A fluorescence of the retina and the liver vitamin A stores or the blood levels was found, at least by the use of the histologic method which may conceal slight differences. Only after the eighteenth week was there apparently a slight decrease of the vitamin A fluorescence of the retina.

The female rats lose their vitamin A stores slower than the males, as is apparent from the chemical assays and histologic pictures. This sex difference is clearly shown if, as in this experiment, a uniform vitamin A supply has been administered during the feeding periods. The type of histologic picture seen during depletion is the same for the two sexes. Since in male rats a lower level is reached faster, the histologic changes occur more rapidly also. The higher blood level in male rats (Brenner et al., '42) cannot thus be explained by the histologic distribution in the liver.

SUMMARY

1. The histologic distribution of the vitamin A fluorescence in the liver was compared with the amount of vitamin A found chemically in liver and blood serum of rats first given large doses of vitamin A, and then placed on a vitamin A free diet. The vitamin A fluorescence ran parallel with the amount of vitamin A found chemically in the liver in all ranges of vitamin A content except when the largest amounts of vitamin were present where the differentiation in the degrees of fluorescence was less clear. This gave additional evidence for the value of the histologic method and recommended it as a simple technic for the estimation of the vitamin A content of organs. No simple parallelism existed between the vitamin A fluorescence of the liver and that of the retina or the blood vitamin A level.

2. Under conditions of large vitamin supply and advanced depletion there was a preponderance of vitamin A in the Kupffer cells over the liver cells. In the middle phase of the depletion period when the amount of vitamin A present compared favorably with that found in adult stock animals, much vitamin A was in the liver cells which probably represent the physiologic storage place. In hypervitaminosis the Kupffer cells store the excess of vitamin A and apparently destroy it; this may explain the uneconomic utilization of vitamin A under conditions of large supply. In depletion the Kupffer cells distribute the remnants of vitamin A.

3. During depletion the livers of male rats lose vitamin A faster than those of females. There is, however, no sex difference with respect to method of utilization, as judged from the histologic picture.

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PLATE 1

EXPLANATION OF FIGURES

Fluorescence photomicrographs of unstained frozen sections of livers, cut after short formaldehyde fixation and mounted in water. The livers are from rats which first received high vitamin A feeding and were then transferred to a vitamin A deficient diet. Magnification of 80. The results of the chemical assay of the liver for vitamin A in Evelyn Photoelectric Colorimeter units per 100 gm. liver as indicated in parentheses in legend are given for comparison (see table 1).

1 Female rat, no vitamin A deficient diet. Enormous vitamin A fluorescence imparted by the Kupffer cells, relatively little by fine lipoid droplets in the epithelial cells (12,931 units).

2 Male rat, 2 weeks on the deficient diet. Strong vitamin A fluorescence seen in the Kupffer cells, moderate in the epithelial cells (5525 units).

3 Female rat, 4 weeks on the deficient diet. Moderate vitamin A fluorescence of the Kupffer cells, relatively much fluorescence imparted by fine lipoid droplets in the epithelial cells (2562 units).

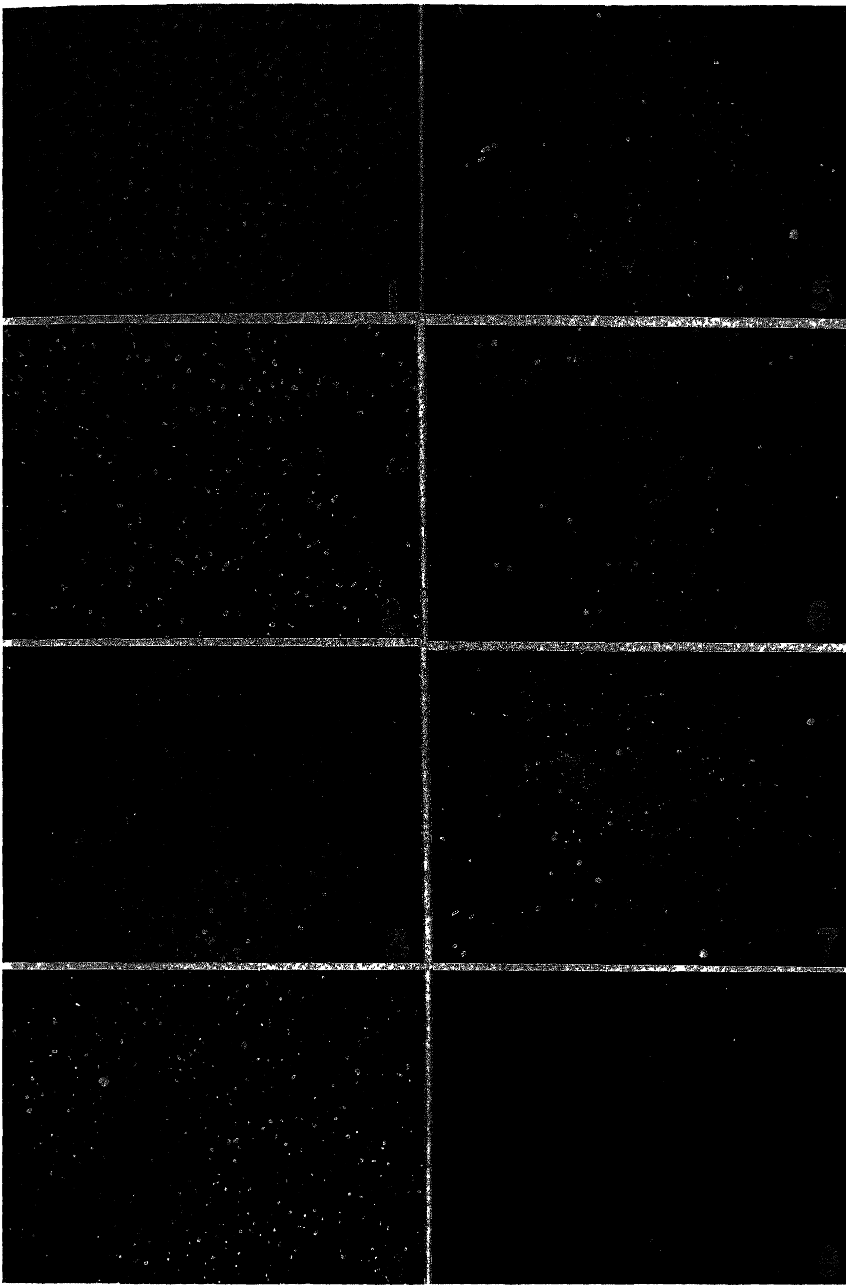
4 Female rat, 10 weeks on the deficient diet. Moderate vitamin A fluorescence of the Kupffer cells and of fine lipoid droplets in the liver cells, distributed close to the Kupffer cells. Homogenous distributions (965 units).

5 Male rat, 10 weeks on the deficient diet. Relatively little vitamin A fluorescence in Kupffer and epithelial cells. Inhomogenous distribution (350 units).

6 Male rat, 23 weeks on the deficient diet. Little vitamin A fluorescence of the Kupffer cells in irregular distribution (80 units).

7 Female rat, 30 weeks on the deficient diet. Moderate vitamin A fluorescence of Kupffer and epithelial cells (449 units).

8 Male rat, 30 weeks on the deficient diet. Traces of vitamin A fluorescence localized in scattered Kupffer cells (50 units).



EFFECT OF MANGANESE ON CALCIFICATION IN THE GROWING RAT

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TWO FIGURES

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Numerous experiments have been reported concerning the question of whether cations other than calcium can be deposited in the bones. Some of the results are of interest purely as experimental phenomena, indicating that the calcification mechanism is not absolutely specific for calcium salts; some, however, have shown the fate and to a large extent have guided the therapy of certain toxic metals in the body. Examples of metals used in such studies are lead, strontium, beryllium, radium, bismuth, and manganese.

Manganese, like beryllium, aluminum, and iron, has proved rachitogenic when fed in large amounts (Blumberg, Shelling and Jackson, '38). Since the major part of this element is excreted in the feces (Skinner and Peterson, '30), the harmful action is probably caused, not by the toxicity of manganese itself, but by its effect on phosphorus metabolism. Because appreciable amounts of manganese itself are absorbed and found in various tissues, including bone, there is also the possibility that it may have a direct influence on the calcifying mechanism.

Recent work has indicated that traces of manganese (25 to 50 p.p.m.) in the diet of chicks prevent perosis (Gallup and Norris, '39 a). A requirement as definite as that suggested for the chick has yet to be demonstrated in the rat, although manganese appears to have an important role in the produc-

tion and rearing of normal young of both rats and chickens (Gallup and Norris, '39 b; Orent and McCollum, '31; Barnes, Sperling and Maynard, '41).

The ingestion of certain acidic organic compounds has been reported to influence the cure of rickets by improving mineral absorption (Hathaway and Meyer, '39; Shohl, '37). Since manganese complexes with organic acids were observed by Delsal ('38), the effect of citrate on the mineral elimination in the feces was believed to constitute an important phase of any series of manganese metabolic studies.

The present investigation deals with three problems in manganese metabolism: (1) the effect of large manganese intakes on the fecal output of calcium and phosphorus and on the amount and location of the stored manganese; (2) the effect of phosphate, vitamin D, and citrate on both mineral excretion in the feces and the cure of rickets in the presence of manganese; (3) the probable role of very small manganese intakes in calcification.

EXPERIMENTAL

The experimental work with manganese in the diet was divided into two parts, the first dealing with high manganese intake and the second with small amounts of manganese.

High manganese levels

Young rats weighing between 45 and 50 gm. were depleted of their body stores of vitamin D by feeding some groups the Steenbock and Black ration no. 2965, and other groups the same ration with the calcium carbonate molecularly supplemented or replaced by manganous carbonate. The rachitogenic diets supplemented with manganese are designated as 1/5 S, 2/5 S, 3/5 S, and 5/5 S; while the rations in which the calcium was replaced by manganese are designated as 1/5 R, 2/5 R, 3/5 R, 5/5 R, 2/5 Rp, and 5/5 Ra. Diet 2/5 Rp had the phosphorus content increased by the addition of 1% potassium dihydrogen phosphate; in diet 5/5 Ra, 0.5% of the calcium carbonate was left in the diet (similar to diet C of Blumberg,

Shelling and Jackson, '38). The calcium, phosphorus, and manganese contents of these diets on a dry basis were as follows:

DIET	Ca %	P %	Mn %	DIET	Ca %	P %	Mn %
Steenbock	1.48	0.36	0.002	1/5 R	1.31	0.36	0.42
1/5 S	1.50	0.36	0.312	2/5 R	1.12	0.36	0.68
2/5 S	1.52	0.36	0.66	2/5 Rp	1.16	0.80	0.66
3/5 S	1.55	0.32	1.12	3/5 R	0.93	0.33	1.02
5/5 S	1.40	0.32	1.73	5/5 R	0.08	0.40	1.58
				5/5 Ra	0.84	0.51	1.22

All animals were placed in a darkened room in individual metal cages provided with raised screen floors. They were given ample rachitogenic diet and distilled water. A record was kept of food intake and change in body weight.

After a 21-day depletion period, half of the animals on each diet were killed by anesthesia in order to obtain blood and bones for analyses. The remaining animals were given (1) 5 units of vitamin D (U. S. P. XI reference cod liver oil), (2) 5 units of vitamin D with potassium citrate in the diet (0.4 mols per 100 gm.), or (3) 100 units of vitamin D. At the end of a 7-day curative period, these animals also were killed.

The blood samples obtained by cardiac puncture were pooled for each group of animals. Serum inorganic phosphorus was determined by the method of Youngberg and Youngberg ('30) and calcium by the Clark-Collip modification of the Tisdall method ('25). Livers were pooled for each group of animals. These were dried at 100° C., ground in a glass mortar, and ashed. The ash was analyzed for manganese by the periodate method of Willard and Greathouse ('17).

The feces were collected from individual animals during the 21-day depletion period and during the 7-day curative period. The dried fecal pellets were weighed and pulverized. Duplicate samples were ashed in platinum crucibles. The ash was dissolved in 5 ml. of nitric acid (1:4) by the addition of 1 to 2 ml. of hydrogen peroxide (3%) and the acid solution washed through a quantitative filter paper into a 100 ml. volumetric flask and diluted to volume with distilled water. Aliquots of this solution were used for analysis.

Phosphorus was determined by the volumetric method with ammonium molybdate precipitation (Hillebrand and Lundell, '29, p. 567) and calcium by permanganate titration of the oxalate in the presence of acetic acid (Ibid, p. 501). Manganese was determined by the periodate method, colorimetric measurement being made with an electrophotometer using a green filter with maximum absorption of 525 mμ.

Low manganese levels

The effect of small amounts of manganese on calcification was studied in the following experiments.

1. The Steenbock diet was supplemented with 0.005% and 0.01% manganese added as the carbonate.

2. The Steenbock diet was supplemented with tri-weekly subcutaneous injections of manganese lactate solution. The injections were made at levels of 2.5 mg. and 5.0 mg. per week; these amounts are equivalent to those in 50 gm. of diets containing 0.005 and 0.01% of manganese respectively. A level of 50 mg. per week was fatal 14 hours after the first injection. This high level was tolerated by adult rats, but their weight remained stationary, and after 2 weeks they were in very poor condition.

3. A non-rachitogenic, manganese-free, but otherwise adequate diet was used as a control. It consisted of whole milk powder [1] with supplements of 70 mg. of iron and 15 mg. of copper per week. No manganese could be detected in the ash of a 20-gm. sample of this milk powder by the method used. Manganese was added to the diet as a manganous sulfate solution containing 2.5 mg. manganese per milliliter. This was fed at levels of 1.25 and 2.5 mg. per week, representing 0.0025 and 0.005% dietary levels at a food intake of 50 gm. per week. The solutions of ferric chloride, copper sulfate, and manganous sulfate were mixed with 30 gm. of milk powder and dried. After the animals had consumed this mixture, they were allowed to eat the whole milk powder freely the remainder of the week. The groups were designated as MP, MP .0025, and MP .005.

[1] Klim.

The groups of young rats receiving these diets and supplements were subjected to the same procedures for the depletion and curative periods as described for the animals on high manganese levels with the exception of the rats in the milk powder groups. These animals received no vitamin D and were kept on the diet for 5 weeks.

RESULTS AND DISCUSSION

The average values for the results obtained from each group of animals are given in tables 1 and 2 and figures 1 and 2.

The growth response (table 1) indicated clearly the retarding effect of high manganese intake on growth in rats receiving the Steenbock diet. Considering the effect of manganese supplements with a high calcium-low phosphorus diet, it was evident that the growth of the animals decreased as the amount of manganese in the diet was increased (fig. 1). With equal amounts of calcium and manganese, the animals lost weight during the entire experimental period, losing about 30% of their initial weight in 4 weeks.

The condition of the animals may be explained by the abnormal calcium-phosphorus metabolism induced by the Steenbock diet itself and intensified by the presence of large amounts of manganese. The calcium excretion was increased with increased manganese intake (table 1). The phosphorus excretion was not only increased, but at the higher manganese levels, negative phosphorus balances were indicated by the fecal excretion of amounts of phosphorus greatly in excess of the dietary intake.

The replacement of calcium with manganese without increasing the actual mineral content of the diet produced a similar effect on growth and mineral absorption (table 1; fig. 2). Because a partial replacement of the calcium lowered the calcium content toward a more normal calcium-phosphorus ratio, the condition was less severe. The presence of manganese was still sufficient, however, to give a negative phosphorus balance.

TABLE 1

Average food intake, weight change, and mineral balance of the groups of rats receiving high manganese diets.

DIET	NO. OF RATS	FOOD INTAKE (DRY)	MINERAL INTAKE MG./RAT/DAY			VITAMIN D INTAKE	CHANGE IN WEIGHT	SERUM MG./100 ML.		BONE ASH	LINE TEST[2]	PER CENT INTAKE EXCRETED IN FECES			MANGANESE IN	
			Ca	P	Mn			Ca	P			Ca	P	Mn	Bone ash	Liver mg./100 gm. (dry)
21-day depletion period																
		gm.				*units*	*gm.*			*%*					*%*	
Steenbock	80	108	75.5	18.4	0.1		20.9	11.9	3.6	34.5		67.6	76.5	80.0		
1/5 S	24	95	70.5	16.9	14.6		9.3	11.6	4.1	36.5		77.6	83.5	95.9		
2/5 S	42	93	73.0	17.2	31.6		5.9	11.6	3.1	33.8		70.8	102.9	93.4		
3/5 S	25	85	71.3	14.7	51.5		1.6	11.0	3.5	36.9		72.9	125.2	68.9		
5/5 S	8	84	58.8	13.4	72.6		— 7.5		2.3	36.7		76.7	120.1	79.2		
1/5 R	9	93	59.0	16.2	18.8		11.1	13.4	2.6	39.3		69.5	108.6	81.9		
2/5 R	25	93	57.8	15.8	29.9		8.5	11.4	2.6	34.1		56.7	126.5	85.7		
2/5 Rp	4	109	60.2	41.5	34.3		17.3	10.8	3.7	33.3		66.7	61.2	55.6		
3/5 R	9	90	41.8	14.8	46.0		4.1		2.3	37.1		45.4	126.6	76.3		
5/5 R[1]	4	33	2.0	10.0	39.5		—10.0		4.6	44.5		1075.0	113.0	74.4		
5/5 Ra	6	63	24.4	14.8	45.9		— 9.6		2.9	39.8		59.0	85.2	81.2		
7-day curative period																
Steenbock	24	34	80.0	19.4	0.11	5	3.2	12.3	5.5	35.3	1.5	60.7	68.0	81.8	—	0.83
Steenbock	4	31	65.0	15.8	0.09	100	2.7	13.0	4.7	36.6	2.5	56.8	47.5	77.8		
Steenbock	4	44	90.3	21.9	0.14	5 + K Cit	3.3	10.0	6.1	36.0	3.5	25.4	26.4	78.5		
1/5 S	12	31	66.0	15.8	13.7	5	0.9	12.1	3.7	34.6	—0.3	57.2	74.0	86.1		
2/5 S	12	27	60.8	14.4	26.4	5	1.7	12.6	3.4	33.0	—0.8	59.8	84.0	72.4		
2/5 S	4	25	54.8	12.9	23.7	100	0.3		4.6	29.5	—1.8	72.9	104.7	86.1		
2/5 S	4	27	56.0	18.4	26.4	5 + K Cit	2.3		5.3	31.3	0.7	65.5	57.2	64.4		
3/5 S	8	26	62.0	12.8	44.8	5	— 2.1	12.6	3.2	34.8	—1.0	62.8	115.6	62.3	+	
5/5 S	4	22	43.4	9.9	53.7	5	— 6.0		3.3	31.7	—2.0	71.4	121.2	79.4	+	
1/5 R	5	34	63.0	17.2	20.2	5	3.4	12.2	4.5	33.4	—0.6	48.6	87.2	64.9	+	
2/5 R	12	26	40.4	13.0	24.5	5	— 0.3	12.2	4.1	31.3	—1.5	47.7	102.3	76.3	+	2.81
2/5 R	4	24	40.4	12.9	24.2	100	2.2	12.0	4.2	28.8	—1.3	65.7	89.9	67.7		
2/5 R	4	25	51.8	17.4	22.9	5 + K Cit	3.0	12.0	4.6	32.6	1.5	40.8	45.4	72.9		
2/5 Rp	4	39	63.9	44.0	36.2	5	3.0	11.6	6.0	36.8	2.5	34.3	45.9	65.4		1.71
3/5 R	6	27	37.0	13.2	40.9	5	— 2.8	13.0	3.8	34.5	—2.0	45.0	94.7	69.1	+	5.20
5/5 Ra	6	20	24.4	14.7	45.7	5	— 2.0	12.8	2.9	36.4	0.2	42.1	68.7	64.3	0.007	3.92
												5/5 R (for 14 days)				6.83

[1] Values given for 14 days instead of 21 days.

[2] Where a line test of (—1) indicated mild rickets, (—2) moderate rickets, (—3) severe rickets, and (—4) extreme rickets.

TABLE 2

Average food intake, weight change, and mineral balance of the groups of rats receiving low manganese diets.

DIET	NO. OF RATS	FOOD INTAKE (DRY)	MINERAL INTAKE MG./RAT/DAY			VITAMIN D INTAKE	CHANGE IN WEIGHT	SERUM MG./100 ML.		BONE ASH	LINE TEST [3]	PER CENT INTAKE EXCRETED IN FECES			MANGANESE IN	
			Ca	P	Mn			Ca	P			Ca	P	Mn	Bone ash	Liver mg./100 gm. (dry)
21-day depletion period																
		gm.				*units*	*gm.*			%					%	
Steenbock	80	108	75.5	18.4	0.10		20.9	11.9	3.6	34.5		67.6	76.5	80.0		
0.005% Mn	32	116	77.0	19.9	0.37		20.8	12.2	3.3	33.4		77.0	79.5	89.2		
0.01% Mn	48	113	74.5	21.6	0.68		19.2	11.8	3.4	37.1		70.6	67.2	102.9		
0.005% Inj. [1]	4	114	86.0	20.8	0.12		22.5					47.7	54.8	266.7		
0.01% Inj. [2]	6	97	45.2	15.8	0.09		12.2					72.7	83.6	455.6		
MP	4	119	62.8	55.8	....		54.1					49.3	30.5	....		
MP .0025	4	111	58.2	52.0	0.18		53.5					42.8	27.0	55.6		
MP .005	4	113	55.0	49.0	0.36		50.0					51.8	30.0	55.5		
7-day curative period																
Steenbock	24	34	80.0	19.4	0.11	5	3.2	12.3	5.5	35.3	1.5	60.7	68.0	81.8	....	0.83
0.005% Mn	12	38	73.5	18.9	0.35	5	5.4	12.6	5.1	33.5	1.5	62.0	66.7	88.6		0.89
0.01% Mn	16	36	67.7	19.6	0.56	5	4.9	12.0	5.7	36.5	1.5	65.5	63.2	100.0	....	0.99
0.005% Inj. [1]	4	27	54.8	13.3	0.07	5	2.0	13.0	4.0	34.4	—0.1	65.7	90.5	700.0	0.014	1.70
0.01% Inj. [2]	6	31	65.1	15.8	0.09	5	4.0	13.1	3.2	36.1	0.8	55.0	72.2	722.2		2.10
MP	4	56	89.0	79.5	....		21.0	12.2	10.4	56.6	4.0	52.8	27.3	....	....	0.28
MP .0025	4	55	86.8	77.4	0.18		24.0	12.6	10.7	55.8	4.0	54.7	33.1	61.2		0.42
MP .005	4	54	83.5	74.3	0.36		22.0	11.6	11.2	56.0	3.6	50.7	33.6	61.1	....	0.65

[1] Injections averaged 0.36 mg. manganese/rat/day.

[2] Injections averaged 0.71 mg. manganese/rat/day.

[3] Where a line test of (1) indicated just perceptible healing, (2) distinct healing, (3) advanced healing, and (4) normal bone or complete healing.

Addition of phosphate to the diet nullified the action of manganese on phosphorus excretion and improved the appetite and general condition of the animals (diet 2/5 Rp). It is notable, however, that excess of both calcium and phosphorus in this diet allowed a marked increase in manganese retention during the depletion period.

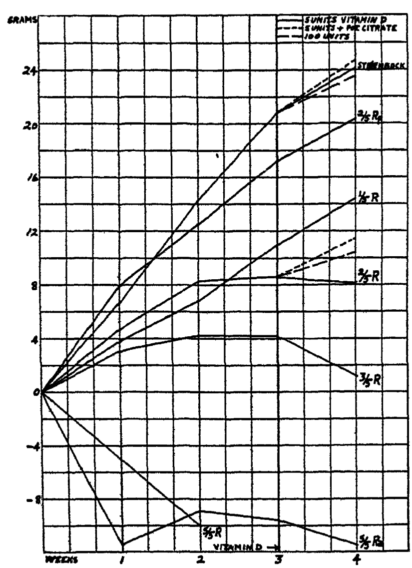

Fig. 1 Growth response in rats in the presence and absence of vitamin D when dietary calcium is supplemented with large amounts of manganese.

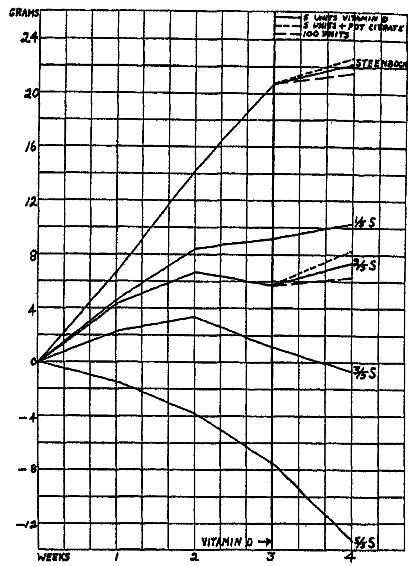

Fig. 2 Growth response in rats in the presence and absence of vitamin D when dietary calcium is partially or entirely replaced by manganese.

The complete replacement of calcium and manganese (diet 5/5 R) resulted in a serious upset in mineral metabolism. Within 14 days some of the animals had died; the others were in very poor condition. The rapid fecal excretion of calcium and phosphorus from the body supply was fatal. Calcium was excreted at an average rate of 21.5 mg. per day, when the intake was only 2 mg. per day. The phosphorus excretion was also greater than the dietary intake, and the serum phosphorus was high.

As a check on this condition, a second group of rats averaging 65 gm. initial weight were fed this diet (5/5 R). The larger animals tolerated the total replacement of calcium no better than the smaller ones. Consequently, 0.5% calcium carbonate was returned to the diet (5/5 Ra) at the end of the first week. The amount of calcium was sufficient to check the rapid loss in weight and to improve the general condition of the animals.

Except in this attempt to replace all of the calcium with manganese, the effect of manganese was more pronounced in relation to phosphorus retention than to that of calcium. Decreasing the calcium to change the calcium: phosphorus ratio also improved mineral assimilation in the presence of manganese. Even when the calcium: phosphorus ratio was reduced so that the diet was no longer rachitogenic, the presence of manganese still induced a rachitic condition.

The administration of vitamin D to animals receiving high manganese diets improved the condition of the animals on all manganese levels, but the effect was most pronounced where the calcium was replaced by manganese. The calcium and phosphorus elimination in the feces was definitely decreased in the presence of vitamin D, and in most cases there was a small gain in weight.

The most striking values in the fecal excretion data for the animals of both the control and the manganese groups are those following the addition of potassium citrate to the diet with the vitamin D supplement. In the animals of the control group and the 2.5 R group the calcium retention was markedly increased; the phosphorus retention was far more than doubled in all three of the groups receiving the citrate (control, 2/5 S, and 2/5 R). The values for phosphorus absorption are given on the assumption that all of the 0.35 to 0.50% phosphorus in the various diets was in available form. Lowe and Steenbock ('36) reported that of the 0.33% total phosphorus in the Steenbock diet only 6% is inorganic, and the rest is in a poorly assimilated form. Hence, the intake values given in these data are maximum. The controls show 23.5 to

32.0% retention of the total phosphorus present in the diet which, according to the work cited, should be about all of the phosphorus available for absorption. Yet with the addition of potassium citrate to the diet, it is notable that almost 75% of the total dietary phosphorus was apparently absorbed. Citrate did not have a marked effect on manganese retention.

Although manganese was evidently instrumental in increasing the fecal excretion of calcium and phosphorus, the feces did not contain all of the ingested manganese. The question arises, was part of the severe condition of the animals caused by the toxicity of the absorbed manganese or by some specific effect on calcium-phosphorus metabolism other than their excretion into the intestine? Marked storage of manganese occurred in the livers of these rats. The amount stored was in proportion to the intake and absorption. It was noted that, although increased phosphorus of the diet (2/5 Rp) resulted in considerable retention of manganese, the amount found in the liver was not as high as from a similar diet without the added phosphorus (2/5 R). The ash of the bones and kidneys contained manganese, but the quantities present in the available samples were too small to allow quantitative measurement by the method used.

In addition to a direct toxic effect of stored manganese, its presence in the tissues may affect the local calcification mechanism in the bone. In the instances of the rats receiving the high manganese diets, both the low blood phosphorus and the results of the line tests indicated a condition of severe rickets. These values, however, are readily explained by the interference with calcium and phosphorus assimilation. With the levels of manganese in the diet that caused a negative phosphorus balance, the phosphorus was drained from both bones and other tissues, and the rachitic condition was probably complicated with osteoporosis. This is especially true with the diet 5/5 R where all of the calcium was replaced with manganese. After only 14 days on this diet, the skeletons of the animals were brittle shells of cortical bone. The epiphyseal line was regular and did not indicate the usual rachitic con-

dition, but the bone had been dissolved from the interior and the marrow cavity filled with blood. The mesothelial linings were hemorrhagic, and the muscles were soft and small. These conditions were undoubtedly caused by the draining of calcium and phosphorus into the intestine. There was nothing which can be interpreted as a specific manganese effect, for the same result could be obtained by maladjustment of the calcium and phosphorus intake alone.

A further indication that the condition was a result of excessive mineral excretion was demonstrated by its improvement with the administration of vitamin D. The general effect of vitamin D was to increase calcium and phosphorus retention and the serum phosphorus level. This increase in phosphorus retention was small but consistent, in spite of the fact that the manganese present was antagonistic to the increased absorption induced by vitamin D. The bones from several groups of animals fed manganese showed some signs of healing along the epiphyseal line, although there was no deposition in the decalcified area of the metaphysis as in normal healing.

Here again the potassium citrate supplement with vitamin D gave significant results as was expected from the improvement in growth and in calcium and phosphorus retention. With the citrate the blood phosphorus was increased and the bones healed to an extent greater than that caused by 100 units of vitamin D alone.

The effects of small amounts of manganese in the diet were not so outstanding (table 2). When added to the milk powder diet, manganese at levels of 25 and 50 p.p.m. showed no appreciable or constant effect on growth, calcium and phosphorus absorption, or blood and bone content. Under these conditions, an average of about 40% of the ingested manganese was apparently absorbed compared to 25% absorption from high manganese intakes when fed with the Steenbock diet. Liver data indicated storage of manganese in proportion to the level in the diet, but the livers of the rats receiving the manganese-free milk powder diet still contained appreciable manganese after 5 weeks' experimental period. About 70% of the total

phosphorus was retained compared to 25 to 30% from the Steenbock diet. The blood and bone analyses yielded normal values.

The groups of rats receiving 50 p.p.m. manganese either by injection or in the diet in combination with the Steenbock diet showed a growth rate, slightly but consistently above that of the control animals. On the other hand, with a level of 100 p.p.m. in the diet, the animals showed slightly retarded growth rates compared to the controls. With the exception of a higher food intake for the animals on the 0.005% levels, the calcium and phosphorus retention and blood and bone analyses showed no appreciable differences between the animals receiving manganese and the control group (table 2).

The injection of manganese in an amount equivalent to 100 p.p.m. in the diet retarded the growth rate to a more marked extent. It may also be seen that the phosphorus excretion was increased in these rats. The explanation for the increased fecal excretion of phosphorus may be in the excretion of the injected manganese, in part at least, into the intestine. The fecal excretion of manganese for both groups in which the manganese was injected was several times the amount ingested in the diet. This agrees with reports in the literature on the excretion of manganese in the bile (Reiman and Minot, '20). In the present studies the amount of manganese found in the livers of the animals injected was twice as great as when the same amount was ingested in the diet.

The injected manganese showed little detrimental effect on the healing of the rachitic condition developed on the Steenbock diet. Vitamin D induced healing in all cases. Even with the manganese injections, the bones of two out of the four animals on the 0.005% level and five out of the six on the 0.01% level showed healing. The serum phosphorus of the latter group, however, remained at a rachitic level consistent with the decreased retention of phosphorus in this group.

From the results of the subcutaneous injections of manganese it appears that the rat does not tolerate more than about 50 p.p.m. of manganese in the diet. At levels of 100 p.p.m. the detrimental effect became evident.

SUMMARY

The effects of various amounts of manganese in the diet of young growing rats were studied with special reference to the influence of this element on calcium and phosphorus metabolism in the presence and absence of vitamin D. The following conclusions were drawn from the data obtained.

1. On high manganese intakes the growth of rats was retarded in proportion to the amount of manganese in the diet.

2. High manganese intake in connection with high calcium-low phosphorus diets increased the excretion of phosphorus in all cases and of calcium at the highest manganese levels. This excretion was by way of the feces and in some cases was increased to the extent of depleting the body supply of these elements. The interference with phosphorus retention was more pronounced than was that with calcium retention.

3. The adverse nutritional condition was less marked when the calcium in the diet was replaced than when it was supplemented by manganese. When the calcium of the diet was completely replaced by manganese, the results were fatal because of the severe loss of calcium and phosphorus from the body.

4. A more favorable calcium : phosphorus ratio in the diet, produced by increasing the phosphorus content, tended to improve retention of these elements even in the presence of manganese.

5. Animals from these groups which had been fed large amounts of manganese developed severe rickets as evidenced by blood phosphorus level and by the condition of the bones. Vitamin D administration resulted in an improved condition.

6. The addition of potassium citrate to the diet produced a striking improvement in calcium and phosphorus retention and subsequently in bone calcification.

7. Manganese was stored primarily in the liver. That which entered the body by subcutaneous injection was stored to some extent in the liver and the bones, but the larger portion was excreted in the feces.

8. Low manganese intakes, comparable to those found effective in correcting perosis in the chick, did not retard

growth and showed little or no effect on bone calcification either in conjunction with normal or with rachitogenic diets.

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THE RELATION OF LIVER STORES TO THE OCCURRENCE OF EARLY SIGNS OF VITAMIN A DEFICIENCY IN THE WHITE RAT[1]

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ONE FIGURE

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Symptoms which have been suggested as evidences of subclinical vitamin A deficiency are nightblindness, lowered vitamin A in the blood, and dryness and roughness of the skin with eruption of papules. Although it is generally recognized that each of these occurs in advanced stages of vitamin A deficiency, there is no agreement as to their relative value in detecting the earliest signs of deficiency.

Since the liver is the body's chief depot for vitamin A, it has been assumed that a certain amount of this vitamin in the liver is necessary to prevent the appearance of these signs. If this is true, a study of the liver stores in relation to the order of occurrence of these several signs would be one approach to the problem of what constitutes the earliest indication of hypovitaminosis A. Since the liver assay is impossible on human beings, it was decided to resort to animal experimentation to throw light on this aspect of the problem.

EXPERIMENTAL

Two series of investigations were undertaken.

Series 1. A study was made of the appearance of the early signs of deficiency in relation to liver stores in 4-week old

[1] Submitted by Sadie Brenner in partial fulfillment for the degree of Doctor of Philosophy in Home Economics at the University of Chicago.

rats. Nine litters (four to eight rats per litter) from mothers fed Sherman's diet 13 were used. Two rats from each litter (one male and one female) served as positive controls. All the rats were fed the vitamin A-free diet [2]. In addition, the controls received butterfat and cod liver oil which were mixed with the diet, the mixture yielding 38 I. U. of vitamin A per gram of food. Individual records of weekly weights and food consumption were kept. One litter was sacrificed each week. Before anesthetizing the animal, the eyes were bleached for 10 minutes by exposure to bright light to insure a maximum concentration of vitamin A derived from the action of light on visual purple (Wald, '35). The body of the animal was then opened, the blood collected, and the liver and eyes removed. It was usually necessary to pool the blood of several animals in order to supply enough for one determination. The whole eyes of all the experimental animals in a litter were pooled and assayed for vitamin A. The livers were analyzed individually. The same procedure was used for the controls. The extracted blood and tissues were analyzed according to the methods of McCoord and Luce-Clausen ('34) and McCoord ('40), using the Evelyn Photoelectric Colorimeter. The macro unit was used for the livers and the micro unit for the blood and retinas. Analysis for carotene was not necessary because previous studies had shown it to be absent from the body of the rats on the diets used. Sections of skin were sent to the dermatology department for histological examination. Measurements of dark adaptation of the animals were attempted but proved to be unsatisfactory due to the lack of a tropistic response of rats to light.

Results of series 1. As shown in table 1, the livers of rats at weaning contained small but measurable amounts of vitamin A (27 units per 100 gm. of liver)[3]. These were entirely lost,

[2] The vitamin A-free diet consisted of 18% alcohol-extracted casein, 4% Osborne and Mendel salt mixture, 1% iodized salt, 67% cornstarch and 10% dried yeast (contributed by the Northwestern Yeast Company, Chicago, Illinois).

[3] Evelyn Photoelectric Colorimeter Units. All liver, eye and blood values refer to these units per 100 gm. of liver and eye tissue and per 100 cc. of serum, respectively, unless otherwise stated.

however, at the end of 1 week of depletion. The livers of the controls, on the other hand, increased steadily in vitamin A.

The blood and eye tissues, however, still contained appreciable amounts of vitamin A even after 7 weeks of depletion. At this time the litter examined had a blood value of approxi-

TABLE 1

The effects of depletion on the vitamin A content of the livers, blood and eyes, and on growth.

WEEKS ON DIET	NO. OF RATS		LIVER		BLOOD [1]		EYES [2]		WEIGHT	
	Deficient	Control	Deficient	Control	Deficient	Control	Deficient	Control	Deficient	Control
	gm.	*gm.*	*units/100 gm.*		*units/100 cc. serum*		*units/100 gm.*		*gm.*	*gm.*
0 [3]	4	4	27	27	8.5	8.5	49	49	40	40
1	5	2	0	236	..	..	107	175	61	59
2	5	2	0	242	12.4	..	68	78	65	91
3	4	2	0	789	10.1	..	95	56	70	118
4	6	2	0	786	9.6	..	36	88	74	143
5	5	2	0	947	7.2	8.6	32	56	75	157
7 [4]	6	6	0	1149	4.7	15.5	44	93	78	199

[1] Blanks indicate results which were rejected due to the formation of a cloudy solution when $SbCl_3$ reagent was added to the evaporated petroleum ether residue.

[2] Two things must be borne in mind in interpreting the data obtained on the vitamin A content of the eyes: (a) the small amount of biological material available; (b) the dependence of the vitamin A content of the eye upon its light adaptation (Wald, '35). The number of eyes used for a single determination was at least four. In order to have a sufficiently concentrated extract of the eye tissues little solvent was used for grinding and washing; consequently the extraction of the tissues may not have been complete. Although the animals in this experiment were placed in the bleaching box for 10 minutes prior to their killing, this does not insure that all the animals were bleached to the same extent due to their varied activity. With these considerations in mind it is believed that the actual figures presented for the eyes can only be used to indicate trends or relative changes.

[3] One litter was used for the analyses of the experimental and control groups at zero weeks on the diet.

[4] Three litters of rats were sacrificed at this time.

mately one-half (4.7 vs. 8.5 units) and an eye content only slightly below that found in the first litter sacrificed (44 vs. 49 units). The blood and eyes of the litter mate controls at 7 weeks showed considerable increases in vitamin A above that found in the 4-week-old rats.

The weight gains of the experimental animals were slight in comparison with those of the controls. The deficient rats continued to gain up to the fifth week of depletion at which time a growth plateau was reached.

A microscopic study of the skin sections [4] revealed no difference in either the histological structure of the stratum corneum or the cutaneous glands of the control and experimental animals. The external appearance of the fur was also not markedly different in the two groups of animals. Even in one litter of rats allowed to die of vitamin A deficiency there were no measurable changes in the histological structures of the skin.

The signs of avitaminosis A which were present in the depleted animals were mild. The rats were small in size as compared to the controls; some of them exhibited a thinning of the fur on the head and slight inflammation and crustiness of the eyelids. Few rats showed difficulty in walking. Only one death occurred within 7 weeks, and that on the thirty-first day of depletion.

This experiment, then, showed that in the absence of liver reserves the rat is able to maintain appreciable amounts of vitamin A in the blood and eye, and to make slight gains in weight for several weeks. Due to the rapid loss of the liver stores, however, the purpose of this experiment, namely, to study the relation of liver stores to the order of the appearance of the early signs of vitamin A deficiency, was unfulfilled. Therefore, a second investigation was undertaken in which 4-week-old rats were made hypervitaminotic with vitamin A concentrate and the effects of subsequent depletion studied.

Series 2. Ten litters of rats 28 to 34 days of age were maintained on their stock diet, and fed approximately 14,000 I. U. of vitamin A [5] daily for 35 days. The supplement was admin-

[4] Dr. S. W. Becker of the Department of Dermatology of Billings Hospital studied the skin sections which were placed in Bouin's fixative, cut from paraffin and stained with hemalum, erythrosine and saffron.

[5] A shark liver oil concentrate furnished by Vitamins Inc., Chicago, Illinois, and halibut liver oil contributed by Mead Johnson and Company, Evansville, Indiana, were used.

istered orally from a calibrated dropper. Two comparable animals received an equal volume of cottonseed oil for the same length of time.

One litter, containing two males and two females, was sacrificed 24 hours following administration of the last dose in order to find the storage resulting from massive feeding. The other nine litters were placed on the vitamin A-free diet. One of these, also containing two males and two females, was sacrificed after 3 days of depletion to determine the rapidity in the rate of loss of liver stores. The remaining eight litters were then classified into twelve groups. Each group, except the last two, contained two males and two females. The basis of selection was such that the average age, weight, and representation from the different litters in the groups were as alike as possible. One group was sacrificed each week for the first 6 weeks of depletion; the time interval between observations was then lengthened in accordance with the rates of depletion. The entire depletion period lasted for 30 weeks, during which time fifty-three animals were examined. Chemical analyses for vitamin A were made of the liver, blood and eyes [6] as in the earlier series. In addition histological examinations of the livers and retinas were made by fluorescence microscopy (Popper and Brenner, unpublished).

Results of series 2. As shown in table 2, the average vitamin A content of the livers and blood of the animals sacrificed 24 hours following the cessation of massive feeding was large in comparison with that found in the stock animals which received cottonseed oil. Throughout the first week of depletion the liver content of the animals sacrificed showed no reduction [7]. Thereafter the liver stores decreased rapidly. In the males 63% of the original stores was lost by the end of the second week, and 93% by the eighth week. After that the loss was more gradual, 98% of the original stores being lost only

[6] These eyes were bleached for 30 minutes.

[7] Since the storage ability of the individual animals varied somewhat, an average of the liver values for the first three groups was used to express the amount of vitamin A storage resulting from massive feeding. The calculations which follow are based on this figure.

at the end of the eighteenth week. The amount remaining in the liver was representative of that found in adult stock animals. The results for the females were similar except that they had a higher vitamin A liver content than did the corresponding males in all groups but one.

The effects of depletion on the blood were more immediate than on the liver stores. Within 3 days the blood level had

TABLE 2

The effects of depletion on the average vitamin A content of the livers, blood and eyes of hypervitaminotic rats[1]

DEPLETION TIME	WEIGHT		LIVER WEIGHT		VITAMIN A CONTENT: Livers				Blood[2]		EYES
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	
	gm.	*gm.*	*gm.*	*gm.*	*units/100 gm.*		*units/total liver*		*units/100 cc. serum*		*units/100 gm.*
0 days[3] cotton-seed gp.		164		6.485		252		16		10.5	
vit. A gp.	191	135	8.796	6.753	9,133	12,340	794	833	40.0		
3 days	185	139	9.290	6.407	7,688	15,649	764	1003	18.7		
7 days	201	163	8.398	6.791	13,078	17,096	1105	1222	18.7		
Average	192	146	8.828	6.650	9,966	15,028	888	1026			
2 wks.	194	155	8.491	6.351	3,663	6,103	303	388	13.2	11.5	83
3 wks.	278	168	10.625	6.230	5,088	4,419	534	276	19.8	9.4	
4 wks.	246	167	8.941	5.962	1,669	3,750	218	150	14.7	8.8	70
5 wks.	251	196	8.619	6.539	1,836	3,783	162	247	17.0	13.1	69
6 wks.	255	173	8.446	5.908	849	3,248	72	182	13.5	7.7	98
8 wks.	314	190	10.089	5.593	684	1,883	69	106	12.4	8.8	35
10 wks.	299	191	9.697	5.418	294	1,115	29	61	15.4	8.6	27
13 wks.	256	181	6.864	5.213	170	1,661	12	85	10.5	6.5	28
18 wks.	272	179	7.366	5.532	159	433	12	24	8.4	6.6	40
23 wks.	270	202	6.926	6.778	80	211	6	14	8.7	6.5	28
30 wks.	322	191	8.028	5.801	50	311	4	20	8.4	8.7	36

[1] Each figure represents the average for the two animals of the same sex except in the last two groups where there are one male and one female, and one male and two females, respectively.

[2] In the first three groups the blood samples of the males and females were pooled so that no separate values are available for the sexes for these periods.

[3] Determinations were made 24 hours after the last dose.

dropped from its initial value of 40 to 18.7 units. Thereafter it continued to fall, but at a much slower rate, until a plateau was reached at about the thirteenth week. If the first high blood value is discounted on the grounds of incomplete removal of vitamin A by the liver, then the average blood level is seen to have decreased from 18.7 to 8.4 units for the males at the thirtieth week of depletion. The average liver stores for that same period dropped from 9,966 to 50 units. The findings

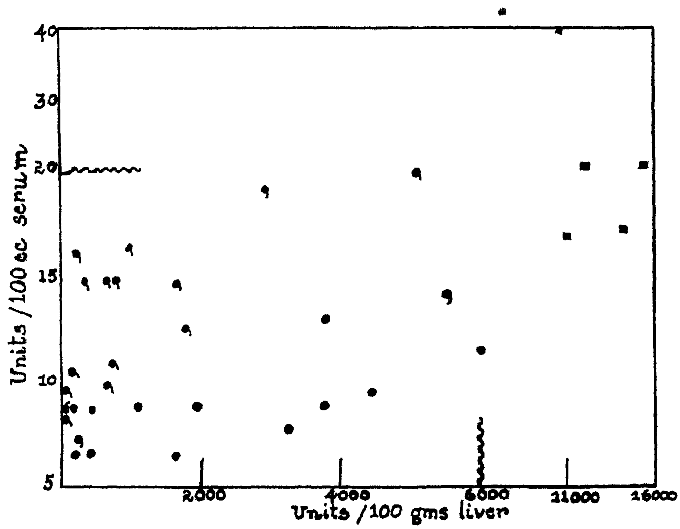

Fig. 1 The relation of blood level to liver stores. Blood values of the males in most cases represent unpooled blood and are plotted against their corresponding liver values; the blood values of the females are usually from the pooled blood of the two females in the group and therefore plotted against their average liver stores. Tagged dots, males; Untagged dots, females; Squares, pooled male and female values.

in the females were again similar but in practically all cases the males had higher blood levels than did the corresponding females.

To determine more clearly the relation between liver stores and blood values, the liver and corresponding blood values for each sex were plotted (fig. 1). A slight tendency toward higher blood values with increasing liver stores during the

early periods of depletion was revealed, especially in the males. However, any one particular blood level covered a wide range in liver stores and, conversely, one liver value was accompanied by a wide range in blood level. For example, a blood level of 8 to 9 units was found with liver stores from 50 to 3,750 units, while a liver storage of approximately 500 units was associated with blood values of 6.5 to 14.9 units.

The vitamin A content of the retina was 83 units at the end of the second week of depletion. At the eighth week there was a decided decrease to 35 units. This approximate level was maintained for the duration of the experiment. A similar amount was found in the eyes of the rats of series 1 after 7 weeks of deficiency.

In this series, the following definite sex differences were observed in respect to storage of vitamin A, rate of depletion and blood levels: (1) On equal intakes, the females had a greater capacity for storage, the males storing two-thirds the amount of the females on the 100 gm. basis. Similar results are obtained on comparing the content of the total livers, though here the difference is not as marked because of the larger livers of the males. (2) The females not only stored more vitamin A, but also retained it more tenaciously (table 2). At the end of the sixth week the males had lost 91% and the females 79% of their original stores; by the end of the thirteenth week the losses were 98% and 91% respectively. At the thirtieth week the livers of the females were found to contain 311 units per 100 gm.; those of the males only 50 units, or approximately twice that in stock rats at weaning. (3) In keeping with their greater capacity for storage and greater ability to retain their stores, the females also had lower blood levels than the males, suggesting a more conservative utilization of vitamin A by the various organs supplied by the blood. To determine the significance of these findings Fisher's t-test was used and the difference found to be significant; these sex differences would have occurred by chance less than one time out of one hundred.

DISCUSSION

Liver stores in 28 day-old rats of diet 13 stock animals disappeared within 1 week whereas the stores of hypervitaminotic rats were still partially filled after 30 weeks on a depletion diet. These rapid losses in young rats have not heretofore been reported. Horton et al. ('41) found almost complete loss in 3 weeks, and Popper and Greenberg ('41) in 2 to 3 weeks depending on the weaning age. Such discrepancies may be explained partly on the basis of the varying nutritional history of the rats and on the different methods used for analyses of liver stores. The rapidity of loss of the excess liver stores of the hypervitaminotic animals confirms the early work of Davies and Moore ('35). The rates of loss in their study were identical with those found in this study through the eighth week of depletion; from then on the rate was slower in the present study. Moore ('40), in later work, failed to confirm the rat's lack of economy in utilizing its excess stores. He attributed the rapid loss obtained previously to a lack of vitamin E in the depletion diet since vitamin E seems to influence the vitamin A storage in the liver (Moore, '40; Bacharach, '40). In the present experiment the animals were kept on diet 13 for the length of the entire supplementary feeding period and it would be expected that enough vitamin E was stored so that the early phase of depletion was not affected.

Although the vitamin A content of the blood and eyes of hypervitaminotic rats decreased from their original values during the process of depletion, the ability of the organism to maintain significant amounts of vitamin A in these tissues in spite of continued absence of liver stores was demonstrated over a period of several weeks. This confirms quantitatively the work on the retina by Greenberg and Popper ('41) who used fluorescence microscopy. In the light of these findings one may question the value of dark adaptation as an indication of one of the early signs of vitamin A deficiency.

The persistence of vitamin A in the blood and eyes, in addition to the small gains in weight made by the animals with no liver stores, suggests the possible presence of vitamin A

in a usable form in other body tissues, thus helping to keep vitamin A in the blood and eye during depletion until these "emergency" stores are used up. This agrees well with Dann's work ('32, '34) which showed that there was no correlation between the vitamin A liver stores present at weaning and the survival time. He suggested a tissue vitamin A not as yet chemically detected. Popper and Greenberg ('41) have demonstrated that the kidneys and adrenals are the only organs other than the retina which retained vitamin A in the absence of liver stores. Horton et al. ('41) did not find vitamin A in the blood when liver stores were empty.

In this study the blood level was found to be of slight value as an index to the nutritional status of the rat except perhaps in extreme deficiency and immediately after massive feeding. These states of vitamin A nutrition were accompanied, respectively, by extremely low and high blood levels. The diagnostic value of intermediate blood levels was by no means clear-cut. All that could be concluded was that higher blood values than those obtained in extreme deficiency, although they tended to be somewhat proportional to liver storage at the upper ranges, can only be interpreted as meaning that stores are present in other parts of the body (i.e., kidney and adrenals) and not as indicating the quantity of reserves in the liver. Closer relationships were obtained between the blood level and liver stores by Horton et al. ('41) and Lewis, Bodansky et al. ('41). A possible cause of these discrepancies is found in Leong's work ('41) which showed that the type of relationship depended upon whether the graded feeding or the depletion technique was used. Positive correlations were obtained with the former, no correlation with the latter.

Sex differences in the vitamin A content of the liver and blood and in the rate of loss of liver stores have previously been reported. From her analysis of the figures of Lagoras and Drummond on control animals, Kimble ('39) has pointed out a sex difference in favor of the females in the ability to store vitamin A. She has also found in human beings that males have a significantly higher blood level than females.

Murrill et al. ('41) have substantiated this conclusion. Bult and Sorgdrager ('38) found a slower rate of loss of vitamin A stores in female and castrated male rats than in normal males. The higher blood level and lower vitamin A stores of the rats may be taken as an indication of a more rapid dispersal of the liver stores, in contrast to the greater economy in utilization by the female. This is not surprising in the light of the role of the female in reproduction and lactation.

It would seem then from this study, that in the rat the use of the vitamin A content of the blood and retina or changes in the histological structure of the skin as early detectors of vitamin A deficiency is not warranted if by deficiency is meant the lack of liver stores. All three of these measurements were resistant to change even in the absence of liver stores. However, it was shown that, although a blood value for a particular rat could not be used to estimate the quantity of its liver stores, the blood value might have some diagnostic significance for large numbers of rats, where in general it could be assumed that higher blood levels were more apt to be associated with higher liver storage than were low blood levels.

SUMMARY

A study of the vitamin A liver stores in relation to the appearance of several alleged subclinical signs was undertaken in rats on a vitamin A-free diet. It was found that:

(1) Whereas 4-week-old rats lost their total vitamin A liver stores after 1 week, hypervitaminotic animals lost 90% of their excess stores after 8 weeks and 98% after 18 weeks. At the thirtieth week of depletion the liver still contained vitamin A.

(2) The vitamin A content of the blood declined rapidly from the hypervitaminotic level, and then decreased more gradually until a plateau was reached for both sexes at about the thirteenth week.

(3) The vitamin A content of the retina decreased to the eighth week of depletion when a plateau was reached.

(4) In the absence of liver stores for several weeks vitamin A was still present in the blood and eye in amounts found in stock rats of weaning age.

(5) A significant sex difference existed. The females stored and retained more vitamin A in the liver than the males, while the males had higher blood levels.

ACKNOWLEDGMENT

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FACTORS INFLUENCING THE ONSET AND CURE OF NUTRITIONAL MUSCULAR DYSTROPHY

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In an earlier paper (Eppstein and Morgulis, '41) we reported our findings, in agreement with those of Mattill ('40), demonstrating the ineffectiveness of dl-α-tocopheryl acetate for curing nutritional muscular dystrophy of rabbits when administered parenterally. The question was raised, whether this failure was due to the lack of an esterase within the body to liberate the free tocopherol, as suggested by Mattill ('38), or whether it was due to the slow rate of absorption of the oil solution of the vitamin, the animal succumbing to the disease before a sufficient amount of the vitamin becomes available to its tissues. In order to answer this question we have employed in this study a water-soluble derivative, disodium α-tocopheryl phosphate. Further, we endeavored to eliminate the factor of rancidity in the vitamin E-low diet by the use of non-oxidative procedures to remove the vitamin; and, finally, a new technique was devised for following the development of dystrophy by the urinary creatine excretion.

METHODS

As in our previous work young albino rabbits were employed. The vitamin E-deficient diet, XP, is a synthetic mixture containing the major foodstuffs, as well as minerals and roughage, in amounts approximating those found in commercial complete rabbit foods. Vitamin B complex was supplied by a rice bran concentrate,[1] and vitamin K by 2-methyl,

[1] Vitab, type II.

1, 4-naphthoquinone,[2] both these materials being incorporated directly in the food. Vitamins A and D were supplied by a weekly supplement, per os, of 0.1 ml. of a concentrated preparation.[3] Unsaturated fatty acids were furnished by feeding 1 ml. of linseed oil once a week, irradiated to destroy the vitamin E. For this purpose quartz flasks, about 1¼ inches in diameter, were filled with oil, tightly stoppered, and exposed to the sun (November) for at least 2 weeks. This was followed by irradiation for 6 hours at a distance of 1 inch from a Labarc Mercury vapor lamp.

The casein was extracted seven times with petroleum ether, as previously described (Eppstein and Morgulis, '41). The regenerated cellulose [4] was ground to a suitable fineness in a corn mill. Olive oil, rather than lard or "seed oils," was employed as the source of fat in order to keep down the vitamin E content. To the dry ingredients, thoroughly mixed, an emulsion of the rice bran concentrate [5] and olive oil was added together with sufficient water to make a stiff dough. This dough was then pressed through ¼ inch wire mesh, spread out and dried for about 15 hours at room temperature with the aid of an electric fan. There could have been only very little loss of the B vitamins since the pH of the dough was about 5.0. The pellets thus produced were pleasant to the taste and, although on exposure to air for several weeks no rancidity was detectable by taste or odor, the material was kept in a well-closed can. The diet was made up fresh every 5 days. Reared on this diet the rabbits seemed healthy but did not grow as well as on our other diets (table 1).

The disodium α-tocopheryl phosphate was prepared from natural α-tocopherol [6] essentially by the procedure of Karrer and Bussman ('40). The salt, dissolved in a minimum of water, was precipitated by the addition of 5 volumes of

[2] Kindly supplied by Mead Johnson Company.

[3] Oleum percomorphum 50% (Mead Johnson).

[4] Kindly supplied by the Sylvania Corporation.

[5] See footnote 1, page 473.

[6] We wish to thank Merck and Company for their generosity in furnishing the product.

acetone, then dried first with acetone and finally in vacuo at 80° C. over phosphoric anhydride. The resultant grayish white powder was only slightly hygroscopic. Analyzed for phosphate by the method of Fiske and Subbarow, as modified for the Evelyn photoelectric colorimeter, its P was found to be 5.51 and 5.62%. This may be compared with a calculated value of 5.59% for P in $Na_2PO_3C_{29}H_{49}O_2$. Inorganic phosphate was not detectable in a 4% solution.

The disodium salt of α-tocopheryl phosphate forms an opalescent foamy aqueous solution, which in a 0.944% concentration (equivalent to 5 mg. α-tocopherol per milliliter)

TABLE 1

Diet XP

Extracted casein	100.0
White dextrin	240.0
Regenerated cellulose	60.0
Sucrose	25.0
Salt mixture (Hawk and Oser)	20.0
Vitab, type II	50.0
Olive oil (containing 40 mg. of 2-methyl 1,4-naphthoquinone	20.0
Total	515.0

has a pH of 9.67 (glass electrode). A 5.5% solution of the monosodium salt (prepared by adding the theoretical amount of HCl) forms a white thixotropic gel which easily passes through a no. 26 hypodermic needle. This solution has a pH of 4.2, but does not taste acid, possibly because of its gel texture. The barium and calcium salts are insoluble in water. All three metallic salts form colloidal solutions (and gels) in fat solvents, such as ether or petroleum ether, but are insoluble in alcohol or acetone. The free acid is somewhat soluble in water but much less so when the solution is acidified. The free acid precipitates are stringy white solids.

The solution of disodium α-tocopheryl phosphate (equivalent to 5 mg. α-tocopherol per milliliter) was preserved with a crystal of thymol in a brown vaccine bottle and autoclaved

at 20 pounds pressure for 30 minutes. Intramuscular injection of this alkaline solution caused neither local reaction nor evidence of discomfort. Injections of 1 ml. were given to the dystrophic rabbit, the dose being repeated when the anticipated end of the protective period approached. The period of protection was counted from the time of the injection until creatinuria reappeared. The calculation of the minimal requirements was made as before in terms of milligrams per kilogram body weight per day.

The onset of dystrophy was evidenced by development of the usual clinical signs and in addition by the determination of the ratio of urinary creatine to total creatinine (preformed plus creatine). The finding of Morgulis and Spencer ('36) that dystrophic animals excrete large amounts of creatine during the active or "acute" phase of the disease has been utilized by Mackenzie and McCollum ('40) as a criterion of dystrophy by determining the absolute increase in creatine excreted per day. This procedure is tedious and involves the use of metabolism cages. As a test for the progress of dystrophy such elaboration is quite unnecessary, for, if the increase in creatine output is referable to a breakdown in muscle metabolism, the urine at any given time should reflect the existing muscular condition. The ratio of creatine to total chromogenic material in a given urine sample will disclose an existing creatinuria as readily as, and under certain conditions even better than, the absolute increase in a 24-hour period. The simplicity of this technique makes the test a practical adjunct in studying nutritional muscular dystrophy.

In practice, the test is carried out in the following manner. A sample of urine is expressed from the bladder and sufficiently diluted so that the chromogenic reaction can be accurately determined. Usually the required dilution can be easily estimated from the color of the urine itself. There is no need to measure either the amount of urine or the degree of dilution. Samples of this diluted urine are analyzed for preformed and total creatinine and the ratio: creatine $\times$ 100/ total creatinine, is calculated. The color determinations are

very easily carried out with an Evelyn photoelectric colorimeter, making a slight modification of the regular Folin procedure. Nitrogen digestion tubes calibrated at 50 ml. and capped with glass thimbles are most convenient both for carrying out the autoclaving and for the subsequent color development. Normal values of the creatine ratio vary from 0 to 10. With the onset of the acute phase of dystrophy this ratio jumps to 30 or 40 overnight, and to about 55 or 60 in another day and, finally, to 75 or 80. Unless treatment is instituted before the ratio reaches the value of 70–80 there is little likelihood of a subsequent cure. With adequate and prompt treatment of the diseased rabbits, the ratio falls rapidly and returns to the normal limits within 2 to 4 days.

EXPERIMENTAL

Effect of cod liver oil. Twenty-one rabbits were put on diet XP. Mild dystrophic symptoms occurred in the usual time of 3 to 5 weeks. Nevertheless the animals continued to grow and their dystrophy did not tend to become more pronounced. The creatine ratio remained essentially normal. Occasionally the growth curve would show a plateau or dip down for a few days while symptoms of dystrophy would also become more pronounced. Immediately prior to or during this period the creatine ratio often would rise to values above the normal. Some animals showed no change in the rate of growth at any time. When by the end of the fifth week no active dystrophy appeared, we suspected that perhaps rancidity was an essential contributing factor for its development. Accordingly, nine animals were given daily supplements of cod liver oil (approximately 1 ml. per kilogram body weight) instead of the weekly percomorph oil supplement. As can be seen from the data recorded in table 2 six of these animals became manifestly dystrophic within 6 to 10 days, the other three in 21 to 23 days. The rabbits which were not fed cod liver oil showed no change in condition. On the fifty-eighth day four more rabbits were given the cod liver oil supplement and all became actively dystrophic within 3 to 10 days. The remain-

ing eight animals on the original diet continued to grow, and, except for a slightly awkward gait and occasional spurt of creatinuria, appeared to be in good health. They were sacrificed on the seventy-eighth day when muscle samples were taken for histological study.

Microscopic examination of the sections showed a latent or "subacute" dystrophy varying from mild (comparatively few muscle fibers in a section affected) to severe (most of the fibers affected). The commonest finding was the loss of transverse striations. Reactive cellular regeneration was fairly common, and a mild fibrosis was also occasionally found.

Because the terms "acute" and "chronic," besides their use in a temporal sense, have in pathology acquired very definite meanings, not necessarily related in any way to time, an unfortunate confusion exists. In the present case the rabbits on the non-rancid diet had a "chronic" dystrophy from the temporal point of view, but pathologically most of the animals presented some state of "acute" dystrophy. In view of the established use of these terms by the pathologist it would perhaps be best to use a different system to designate the temporal phases. Thus the animals not receiving cod liver oil could be said to have a latent or cryptic dystrophy which develops into the clinically manifest or overt dystrophy when cod liver oil is given.

This response of the rabbits to the feeding of cod liver oil is in agreement with the report of Morris ('39) and the extended investigation of Mackenzie and McCollum ('40) although the degree of dystrophy found by the latter workers in the cod liver oil free rabbits was far more severe. The simplest explanation of the results would seem to be that the extracted diet still had sufficient vitamin E to prevent severe and general dystrophy but not enough to prevent gradual dystrophic changes, and therefore the clinical picture was not striking. The feeding of cod liver oil, as pointed out by Mattill ('38), allows the development of rancid products in the gut. This may have accomplished destruction of the

residual vitamin E, causing overt dystrophy to develop. However, there may be also an alternative interpretation of these results.

Parenteral administration of disodium α-tocopheryl phosphate. The α-tocopheryl phosphate ester is very effective in producing cures when injected intramuscularly into rabbits showing active dystrophy. Of the twelve animals so treated eight were rapidly cured. Three of the four failures were already in a very advanced stage of dystrophy, their urinary creatine ratios being over 70 before treatment was instituted. The fourth rabbit had a ratio of 60, but by the next day this rose to 71, then on the following day to 74, when the animal died.

The animals cured with α-tocopheryl phosphate showed minimum daily requirements varying from 0.20 mg. to 0.64 mg. per kilogram body weight. The average value is 0.31 mg., or 0.26 mg. if the single unusually high value of 0.64 mg. is not included in the calculation. Two comparative tests were made with orally administered sesame oil solutions of dl-α-tocopheryl acetate [7] equivalent to 10 mg. of tocopherol. One of the animals previously cured with the phosphate ester (minimum requirement 0.43 mg.) now showed a minimum requirement of 0.65 mg. per kilogram. No strict comparison between these requirements can be made, however, because of the small number of animals. Furthermore, although racemic α-tocopherol has been reported to function probably equally as well as the natural product (Karrer, Fritzsche, Ringier and Salomon, '38), still it is well to point out that the tocopheryl acetate we used was the racemic product. Nevertheless, it is apparent that intramuscular injection of the water-soluble disodium α-tocopheryl phosphate is at least as effective as oral administration of the dl-α-tocopherol acetate, in striking contrast to the ineffectiveness of the parenteral administration of the oil soluble acetate ester. This suggests that the difference in behavior of orally and parenterally administered

[7] Kindly supplied by Hoffman-LaRoche, Inc.

TABLE 2

Effect of cod liver oil and tocopheryl esters on the progress of muscle dystrophy.

RABBIT NO.	COD LIVER OIL SUPPLEMENT BEGUN ON . . DAY	ACUTE DYSTROPHY IN . . DAYS AFTER C.L.O. TREATMENT	TOCOPHERYL PHOSPHATE INJECTED (TOTAL)	TOCOPHERYL ACETATE FED	PROTECTED FOR . . DAYS	MINIMUM REQUIREMENT	CREATINE RATIO $\left(\frac{\text{CREATINE} \times 100}{\text{TOTAL CREATININE}}\right)$	COMMENTS
	days	*days*	*mg.*	*mg.*	*days*	*mg./kg./days*		
3	35	6	15				78 at start of treatment; 74 at time of death	Died in 8 days
4	35	9	10		23	0.23	46 at start of treatment; in 3 days down to 10	
8	35	23	12.5		22	0.30	44 at start of treatment; in 3 days dropped to 25, and in 6 days to 9	
11	35	7	5				72, 77 on second day	Died in 2 days
12	35	9	10		22	0.29	58 at start of treatment; down to 8 in 4 days	
15	35	22	15				59 at start of treatment; 74 at time of death	Died on 3rd day
16	35	21	17.5				48 at start of treatment; 12 after 4 days	
19a	35	10	10		16	0.42	54 at start of treatment; 5 after 5 days	
20	35	7	10		29	0.20	41 at start of treatment; 62 next day, and 10 after 2 days	
13	58	6	15		14	0.64	52 at start of treatment; dropped steadily to 9 in 8 days	
17	58	9	10		24	0 20	22 at start of treatment; 37 next day, and 2 on fourth day	
18	58	3	15				68 at start of treatment; 73 at time of death	10 mg. injected first day; 5 mg. second day. Died on third day
14	58	3		10	13	0.65	51 at start of treatment; 7 after 2 days	Fed at one time
19b				10	8	0.77	43 at start of treatment; 1 after 3 days	Fed at one time
1							Creatine ratio wavered between 15 and 25 but returned to normal, about 9	Dystrophy symptoms appeared on 22nd day
2							Creatine ratio high, but still within normal range, for about 2 days; about 24 on 48th and 49th day	Dystrophy symptoms appeared on 28th day
5							Between 64th and 70th day increased up to 40 but dropped back again	Dystrophy symptoms appeared on 19th day
6							Normal whenever tested	Dystrophy symptoms appeared on 22nd day
9							33 on 63rd day but dropped again	Dystrophy symptoms appeared on 20th day
10							Normal whenever tested	Dystrophy symptoms appeared on 26th day
21							Occasional high values (up to 47) with return to normal	Symptoms appeared on 22nd day
22							Normal whenever tested	Symptoms appeared on 17th day. Killed on 61st day because of accidental injury

tocopheryl acetate may possibly be due to a difference in solubility and rate of absorption rather than to a lack of an enzyme necessary to hydrolyze the injected ester.

DISCUSSION

The oil soluble acetate ester of α-tocopherol fed by mouth and the water soluble phosphate ester injected intramuscularly are both effective in curing rabbits which have become severely dystrophic upon the inclusion of cod liver oil in their diet. Mackenzie, Mackenzie and McCollum ('41) using free α-tocopherol under similar experimental conditions found that this had to be fed in very large amounts to produce cures. This may be attributed to the development of rancidity in the intestine when cod liver oil is present in the diet, causing the oxidation and destruction of the free α-tocopherol. The tocopherol esters are known to be stable under conditions where the free alcohol is easily destroyed. While there can be no gainsaying that intestinal rancidity is a factor of considerable importance in the destruction of α-tocopherol, nevertheless the experiments with orally or parenterally administered tocopherol esters do not completely exclude the possibility of an alternative interpretation, that the cod liver oil itself may possibly exert an injurious action (see Morgulis, '38, p. 12) which promotes the dystrophic effect of the E-avitaminosis. This can be decided only by further, especially devised experiments.

We suggest that the water soluble disodium α-tocopheryl phosphate would probably prove itself more suitable than the oil soluble α-tocopherol or its acetate ester now used in clinical studies because of the ease of administration and possibly better utilization. It is quite likely that vitamin E deficiencies develop in some cases not from lack of the substance in the diet but rather from slow and imperfect absorption from the intestines, or even from excessive destruction in the organism. Such conditions could be more effectively remedied by the parenteral administration of an easily absorbable com-

pound, like the α-tocopheryl phosphate. The discordant results from the clinical use of tocopherol in the treatment of certain diseases might be explained, in part at least, on the basis of its variable absorbability from the intestine.

SUMMARY

1. The parenterally administered disodium phosphate ester of α-tocopherol is effective in treating nutritional muscular dystrophy of rabbits in contrast to the ineffectiveness of injected α-tocopheryl acetate.

2. The finding that cod liver oil is necessary in the diet to produce active dystrophy in rabbits has been confirmed and its significance discussed.

3. A simplified and accurate test of creatinuria for detecting active dystrophy has been described.

4. The use of aqueous solutions of the sodium salts of α-tocopheryl phosphate for parenteral administration in clinical treatment of suspected avitaminosis-E has been suggested.

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THE DAILY INTAKE OF ASCORBIC ACID REQUIRED TO MAINTAIN ADEQUATE AND OPTIMAL LEVELS OF THIS VITAMIN IN BLOOD PLASMA [1]

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Neuweiler ('39) estimated that 0.8 mg. ascorbic acid per 100 ml. plasma denoted a good normal value without saturation of the tissues, and this was in agreement with the view of Greenberg, Rinehart and Phatak ('36) that plasma levels of 0.7 to 0.9 mg. ascorbic acid were adequate but not optimal. Farmer and Abt ('38) set 0.7 mg. per 100 ml. as the prescurvy level. In view of these reports the Northwest Nutrition Cooperative Research Group took 0.8 mg. ascorbic acid per 100 ml. as the criterion for judging an adequate state of nutrition as regards ascorbic acid. Their results on the nutritional status of college students with respect to ascorbic acid will be published soon.

In this investigation there have been determined the intakes of ascorbic acid necessary to maintain plasma levels of 0.8 mg. ascorbic acid per 100 ml., as well as saturation levels.

[1] This investigation is part of the regional project of the Northwest States on the ascorbic acid metabolism of college students.

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INTAKES OF ASCORBIC ACID NECESSARY TO MAINTAIN PLASMA LEVELS OF 0.8 MG. PER 100 ML.

Methods

Four college students, two men and two women, 21 or 22 years of age, served as subjects. They were placed on a constant diet practically the same as that used by Belser, Hauck and Storvick ('39) and Todhunter and Robbins ('40). This diet contained weighed amounts of canned beets, carrots, dried prunes, evaporated milk, cheese, and beef; eggs, cereals, butter, nuts and sugar were used ad libitum. Each food was analyzed for ascorbic acid by either the indophenol titration method of Bessey ('38 b), or his photoelectric colorimetric modification of it ('38 a). The prunes and beets were analyzed by the method of McHenry and Graham ('35). This diet provided 8 to 11 mg. ascorbic acid per day. Daily supplements of ascorbic acid [2] were given each morning, directly after a blood sample had been drawn. Plasma ascorbic acid determinations were made daily according to the micro method of Farmer and Abt ('38).

For 3 pre-experimental days a supplement of either a pint of orange juice, or 100 mg. crystalline ascorbic acid was given, in order to be sure that the blood ascorbic acid concentration was above 0.8 mg. per 100 ml. plasma. This guarded against the possibility of the daily ascorbic acid supplement going to make up a previous deficiency. The preliminary period was followed by a 7-day experimental period when the two women received 50 mg. and the two men 60 mg. ascorbic acid per day in addition to the amount contained in the diet. After another 2- or 3-day period on 100 mg. per day to raise the blood level above 0.8 mg. ascorbic acid per 100 ml. plasma, there followed another 7-day experimental period on different supplements of ascorbic acid; and for the women, a third period was carried out some months later.

Results

The blood plasma ascorbic acid values are given in table 1. After each subject had received a constant amount of ascorbic

[2] The ascorbic acid was generously supplied by Merck and Company.

acid for 3 days, the plasma ascorbic acid concentration dropped to approximately a stationary point, providing the plasma level was maintained at approximately 0.8 mg. per 100 ml. or above. The plasma values for the last 3 days on each level of intake are therefore expressed as ranges, rather than being

TABLE 1

Daily blood plasma ascorbic acid levels on different intakes of ascorbic acid.

DAY	SUBJECT MLS — ♀ HEIGHT 154 CM., WEIGHT 45 KG.		SUBJECT LS — ♀ HEIGHT 164 CM., WEIGHT 60 KG.		SUBJECT WL — ♂ HEIGHT 168 CM., WEIGHT 60 KG.		SUBJECT RS — ♂ HEIGHT 176 CM., WEIGHT 75 KG.	
	Supplement to diet	Blood plasma	Supplement to diet	Blood plasma	Supplement to diet	Blood plasma	Supplement to diet	Blood plasma
				Series I				
	mg.	*mg./100 ml.*	*mg.*	*mg./100 ml.*	*mg.*	*mg./100 ml.*	*mg.*	*mg./100 ml.*
1–3	100	0.92–1.06	100	1.14–1.19	100	1.08–1.16	100	0.92–1.05
4	50	1.02	50	1.12	60	1.19	60	1.06
5	50	1.00	50	1.10	60	1.04	60	1.02
6	50	0.89	50	0.95	60	1.03	60	0.85
7	50	0.82	50	0.89	60	1.01	60	0.96
8–10	50	0.82–0.85	50	0.92–0.99	60	0.80–0.82	60	0.87–0.74
11	100	0.82	100	0.86	100	0.84	100	0.66
12	100	1.00	100	1.00	100	1.02	100	0.80
13	40	1.02	40	1.06	50	1.00	170	0.86
14	40	0.99	40	1.03	50	0.95	80	0.87
15	40	0.95	40	0.91	50	0.83	80	0.79
16	40	0.93	40	0.89	50	0.83	80	0.85
17	40	0.82	40	0.86	50	0.82	80	0.80
18–20	40	0.82–0.86	40	0.86–0.90	50	0.76–0.77	80	0.72–0.81
		Series II						
1–2	100	0.94–1.05	100	0.98–1.06				
3	30	0.84	30	0.85				
4	30	0.63	30	0.84				
5	30	0.54	30	0.78				
7–9	30	0.74–0.59	30	0.90–0.64				

given as individual values for these days. The women, when on the 30 mg. supplement, and the man, RS, when receiving 60 mg. ascorbic acid as the supplement, did not show this leveling off. The ascorbic acid concentration of the plasma continued to go down in those cases, apparently showing too

low an intake. The daily intakes (food sources plus supplements) necessary to maintain the level of 0.8 mg. ascorbic acid per 100 ml. were therefore considered to be about 49 mg. ascorbic acid for MLS, 38 mg. for LS, between 59 and 69 mg. for WL and at least 89 mg. for RS. These intakes corresponded approximately to 1.1, 0.8, 1.1 and 1.2 mg. per kilogram, respectively.

One other subject, ER, in a different series reported below, on an intake of 61 mg. ascorbic acid showed a plasma concentration of 0.81 mg. per 100 ml. This amounted to an intake of 1.0 mg. per kilogram. The average requirement with its standard deviation to maintain this blood level was therefore 1.0 ± 0.13.

INTAKES OF ASCORBIC ACID NEEDED FOR "TISSUE SATURATION"

Methods

A similar investigation was begun on four women subjects, to determine the intake of ascorbic acid necessary to maintain tissue saturation evidenced by plasma ascorbic acid concentration and excretion of ascorbic acid in response to a test dose. Unfortunately, one of the student subjects left school before the investigation was completed, but work was continued on the remaining three persons.

The method as described by Belser, Hauck and Storvick ('39) was followed. Each subject was brought to a state of tissue saturation by the daily ingestion of 200 mg. ascorbic acid for 3 or more days. A test dose of 400 mg. was then given and the excretion of ascorbic acid during the next 24 hours measured. The individual was then placed on a constant intake of ascorbic acid, e.g., 60 mg. for 6 days, after which the test dose of 400 mg. was again administered and excretion during the next 24 hours measured. After resaturation again, the test period was repeated with a different supplement, and the test dose once more administered. Blood plasma ascorbic acid determinations were made by the micro method of Farmer and Abt ('38) before each test dose and 24 hours after. With

two of the subjects, daily blood determinations were made through at least two of the experimental periods.

The basal diet, which was the same as that reported above but with different lots of canned goods, provided a total of 11 mg. ascorbic acid per day.

Urine was collected in quart jars in which the following preservative as recommended by Sendroy (private communication) had been placed: 75 ml. 5N sulfuric acid, 2 ml. toluene and 2 ml. 8-hydroxyquinoline (1.45% alcoholic solution). Urine preserved in this way retained 95% of its ascorbic acid. The ascorbic acid concentration in the urine was determined by titration with a solution of 2,6-dichlorophenol indophenol, standardized daily, as well as by Bessey's method ('38 a) using the Evelyn photoelectric colorimeter. There was close agreement between the values obtained by the two methods.

Results

The lowest excretion in the 24 hours after the test dose of 400 mg. ascorbic acid when the subject was known to be in saturation (i.e., after receiving 200 mg. for at least 3 days) was taken as index of saturation for that individual. Likewise the lowest blood plasma ascorbic acid level when the subject was in known saturation was considered as the saturation level for that subject. The values (table 2) for excretion and plasma level per 100 ml., respectively, were as follows for each subject: MF, 272 and 1.0 mg.; AG, 267 and 1.2 mg.; ER, 291 and 1.1 mg.; and LS, 325 and 1.1 mg.

In no case except for subject AG, did the excretion of ascorbic acid in response to the test dose parallel the previous intake of ascorbic acid. For MF and ER, at no time after an experimental period did the excretion after the test dose reach the lowest level of excretion after saturation. After AG had ingested 111 mg. a day for 6 days, the excretion after the test dose amounted to 251 mg., an amount very near to the 267 mg. excreted when she was in known saturation.

In general, the blood plasma levels were higher with the higher intake and paralleled the intake more closely than did response to the test dose. When plasma levels were determined daily, they were quite consistent at any one level of intake. For instance, when MF was ingesting 111 mg. ascorbic acid per day, her plasma values on successive days were 0.87, 0.87, 0.84, 0.88, 0.89 and 0.94 mg. per 100 ml., and when her

TABLE 2

Blood plasma ascorbic acid levels after a constant intake of ascorbic acid for 3 to 6 days followed immediately by a test dost of 400 mg.; and plasma levels and excretion after the test dose.

Daily intake	No. days at that level	Blood plasma levels: Before test dose	Blood plasma levels: After test dose	Excretion in 24 hours after test dose
mg.		*mg./100 ml.*		*mg.*
Subject MF — Height 163 cm.; Weight 61 kg.				
200	3	0.99	1.01	393
200	3	1.10	1.10	318
200	3	1.22	1.06	334
200	3	1.06	0.99	272
61	6	0.63	0.88	237
91	6	0.91	0.97	190
111	6	0.90[1]	0.94	225
131	6	1.05[1]	1.06	223
Subject AG — Height 165 cm.; Weight 66 kg.				
200	3	1.26	1.23	328
200	3	1.31	1.35	267
91	6	1.03[1]	1.14	212
111	6	1.18	1.22	251
Subject ER — Height 165 cm.; Weight 59 kg.				
200	3	1.29	1.22	393
200	3	1.12	...	381
200	3	...	1.19	291
200	3	1.18	1.14	299
61	6	0.81	1.05	236
81	6	0.93	0.99	216
111	6	1.12	1.20	255
Subject LS — Height 152 cm.; Weight 52 kg.				
200	3	1.20	1.19	430
200	3	1.18	1.10	325
61	6	0.89	0.92	279
81	6	0.81	0.88	218

[1] Average of last 3 days.

intake was 131 mg. the daily plasma values amounted to 1.09, 0.92, 1.17, 0.98, 1.00 and 1.06 mg. per 100 ml. It is therefore our opinion that the plasma values represent a better criterion than those for excretion for judging saturation after a test dose.

The daily intakes of ascorbic acid necessary to maintain tissue saturation amounted to 131 mg., 111 mg., and 111 mg.,

respectively, as measured by blood plasma ascorbic acid concentration; these represented intakes of 2.0, 1.8 and 1.7 mg. per kilogram of body weight for the three subjects.

These intakes of ascorbic acid necessary to maintain saturation are comparable with those reported by other workers as follows: Belser, Hauck and Storvick ('39), 70 to 100 mg.; Todhunter and Robbins ('40), 90 to 110 mg.; and Ralli, Friedman and Sherry ('39), 100 mg.; and with the 100 mg. or more estimated by Smith ('38) to constitute a luxus consumption level. These values, ranging from 1.0 to 1.8 mg. per kilogram, with those reported above, 1.7 to 2.0 mg. per kilogram, indicate that there is no relation between body weight and ascorbic acid required to maintain saturation levels in the blood.

SUMMARY AND CONCLUSIONS

The daily intake of ascorbic acid necessary to maintain a blood level of 0.8 mg. ascorbic acid per 100 ml. plasma has been determined for five subjects. This amount was 38–49 mg., 49 mg. and 61 mg., respectively for three women; and 69 mg. and 89 mg. per day for two men, corresponding to intakes of 0.8 to 1.2 mg. per kilogram, averaging 1.0 ± 0.14.

In three different subjects, there were measured the intakes of ascorbic acid necessary to maintain tissue saturation as determined by blood plasma levels. These values amounted to 111, 111 and 131 mg., respectively, or 1.7, 1.8 and 2.0 mg. per kilogram body weight.

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STUDIES ON THE VITAMIN B COMPLEX IN THE NUTRITION OF THE DOG [1]

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TWO FIGURES

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Studies on the vitamin B complex in the nutrition of the dog have been carried on in this laboratory using a purified casein — sucrose ration supplemented with synthetic vitamins and liver extracts. Deficiencies of riboflavin (Axelrod, Lipton and Elvehjem, '40, '41), nicotinic acid (unpublished data), pyridoxine (McKibbin, Madden, Black and Elvehjem, '39; McKibbin, Schaefer, Frost and Elvehjem, '42), pantothenic acid (McKibbin, Black and Elvehjem, '40), and choline (Schaefer, McKibbin and Elvehjem, '41) have been studied or demonstrated. During our earlier studies on pantothenic acid deficiency evidence was obtained for the existence of two additional factors essential for the dog — one stable to alkali, the other labile to alkali. Recently we have shown that this work was probably complicated with choline deficiency (Schaefer, McKibbin and Elvehjem, '41) and that choline was undoubtedly one of these factors. The availability of synthetic pantothenic acid has enabled us to continue the study of the unknown factor or factors.

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EXPERIMENTAL

The basal ration and general experimental procedure have been described previously (Schaefer, McKibbin and Elvehjem, '41). The ration had the following percentage composition: sucrose 66, acid-washed casein 19, cottonseed oil 8, cod liver oil 3, and salt mixture 4. This ration was supplemented with 100 μg. per kilogram of body weight per day of thiamine chloride and riboflavin, 2 mg. of nicotinic acid, 60 μg. of pyridoxine hydrochloride, and 500 μg. of calcium pantothenate. Weanling puppies were used in all of the experiments unless otherwise specified.

During our earlier studies on pantothenic acid deficient rations we observed that animals receiving liver extract which had been treated with ether would not respond consistently to purified concentrates of pantothenic acid (McKibbin, Black and Elvehjem, '40). This suggested that the acid ether extraction procedure removed some other essential factor. It was thought advisable, therefore, to use the acid ether extract or the acid ether residue in basal rations containing synthetic pantothenic acid with the idea of producing an uncomplicated deficiency of either one of the two unknown factors.

Accordingly, four pups were given the basal ration supplemented with 2% of the acid ether residue of liver extract "fraction D."[2] One of these animals received in addition 50 mg. of choline chloride per kilogram of body weight per day. Another pup received 2% of the acid ether extract of fraction D supplemented with choline as above. Of these five dogs, four showed excellent growth and appeared in normal condition over the assay periods of 167, 131, 79 and 77 days. The fifth dog, receiving the acid ether residue unsupplemented with choline, showed complete growth failure by the thirty-ninth day. Growth was resumed when a lead acetate filtrate of liver extract powder 1:20[3] was added to the ration.

[2, 3] The Wilson Laboratories.

From these results it is evident that, although use of these liver fractions can result in a demonstrable deficiency, these fractions are not dependable for assay of liver extracts containing these factors. It is probable that much of the choline in liver extracts is not removed with acid ether since two of the dogs receiving the acid ether residue grew normally without added choline, whereas we have found that on a choline-free ration deficiency can be expected in 3 to 6 weeks.

In our earlier studies we also observed that liver extracts treated with alkali to remove pantothenic acid would not permit growth indefinitely with the periodic addition of pantothenic acid concentrates (McKibbin, Black and Elvehjem, '40). These results suggested that there was an essential factor in liver extract which was labile to alkali. It was therefore decided to study this factor using alkali-treated liver extracts.

Three littermate puppies were used in this experiment. They received the basal ration supplemented with 50 mg. of choline chloride per kilogram of body weight per day. In addition one received 2% of the alkali treated fraction D, another received 2% of the acid acetone extract of fraction D (McKibbin, Madden, Black and Elvehjem, '39), and the last received 2% of this fraction treated with alkali. The alkali treatment of these fractions was carried out by diluting ten times with water, adjusting to neutral with litmus, adding solid KOH to a concentration of 1 N and then heating on the steam bath for 1½ hours at 88–95°C. The preparations were then neutralized with acid, the salts precipitated with alcohol, and the filtrates concentrated under reduced pressure. These dogs all showed excellent growth and appeared normal throughout the assay periods of 115, 204 and 115 days. The comparison of these results with those of our earlier studies indicates that the alkali labile factor encountered was undoubtedly choline.

A choline deficiency (Schaefer, McKibbin and Elvehjem, '41) was then produced on the synthetic ration supplemented only with thiamine, riboflavin, nicotinic acid, pyridoxine, and

pantothenic acid. The puppies used in the choline experiment were then continued on the basal ration supplemented with 50 mg. of choline chloride per kilogram of body weight per day. Dogs 193, 195, 196 and 197 were given this ration while dog 192 was given in addition 2% of liver extract fraction D. The growth of the first four dogs was suboptimal and erratic while that of dog 192 was fairly consistent. After the dogs had received this ration 4½ months without developing an acute

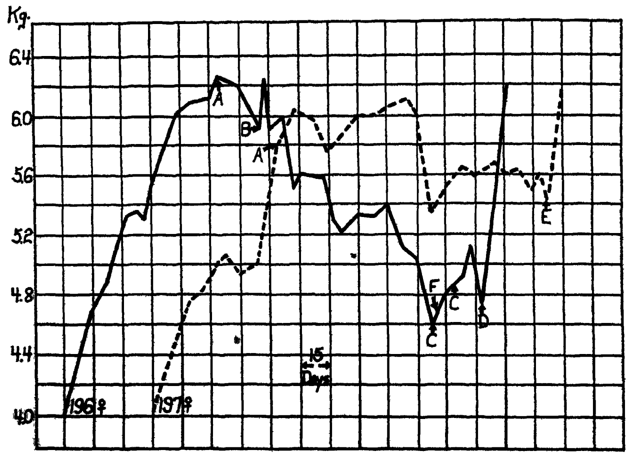

Fig. 1 Response of two growing dogs to the replacement of the acid-washed casein with the alcohol-extracted casein in the basal synthetic ration. A. Changed to purified casein ration; B. Started 50 mg. per kilogram body weight per day of i-inositol; C. Oral administration of i-inositol and p-amino benzoic acid; D. Started 3% liver extract powder 1:20 in ration; E. Started 6–10% whole liver powder in ration; F. Started intravenous injections of glutamine.

deficiency, it was decided to purify the casein further. This was done by refluxing with 95% ethanol for four 12-hour periods. The response of dogs 196 and 197 to this change is shown in figure 1. After 3 weeks there was a slow steady decline in weight until at the end of 3½ months the dogs had lost approximately 25% and 11% of their body weight, respectively. In contrast to this, dogs 193 and 195 maintained on the acid-washed casein ration continued to grow to weights of 6.8 and 7.3 kg. Inositol feeding at 50 mg. per kilogram

per day over a 16-day period failed to prevent this loss in weight of dog 196. Later two oral administrations, each of 200 mg. p-amino benzoic acid and 1 gm. of inositol, along with three intravenous injections of 50-mg. of glutamine in an 11-day period produced only a slight increase in weight. The addition of 3% liver extract powder 1:20 to the basal ration produced a complete and immediate restoration of the lost weight. The addition of 6% and then 10% of whole dry liver to the ration of dog 197 restored the original weight of this dog. Another dog (no. 198) given this purified casein ration for 1½ months also reached a growth plateau. After 3 months more this ration was supplemented with 3% liver extract powder 1:20 which resulted in a weight increase of 0.8 kg. (13% of body weight) in 12 days.

The apparent nutritive differences between the alcohol extracted casein and that used in our previous studies suggested an experiment using this purified casein from the start. Accordingly, a litter of five dogs was placed on this ration supplemented with the six synthetic vitamins at levels used in the previous experiments. One dog (no. 202) received in addition 2% liver extract powder 1:20. The response of these dogs is shown in figure 2. Choline was withheld from dog 207 until deficiency appeared, then it was added to the ration and growth was resumed. It can be seen that only one of the dogs not receiving liver extract grew at a rate comparable to that of the dog receiving liver extract. The others reached a growth plateau 2 to 5 kg. below this weight.

The results with five dogs from various litters placed on the acid-washed casein ration were different from the above results using alcohol-extracted casein. Dogs nos. 186, 188, 190, 191, and 174 received liver supplements for periods of 3, 3, 4½, 5, and 5½ months, respectively. After these periods all liver supplements were discontinued and the ration supplemented only with thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, and choline. These animals have been maintained on this ration in normal condition for 9½, 9½, 4½, 5, and 5½ months, respectively. Of these dogs,

nos. 186, 188, 190, and 191 were subjected throughout these periods to the additional strain of weekly phlebotomy in our hemorrhagic anemia studies and they are still in excellent condition.

In some growing dogs, however, the acid-washed casein ration supplemented with synthetic vitamins frequently shows failure to support growth. Dog 172 receiving all six vitamins

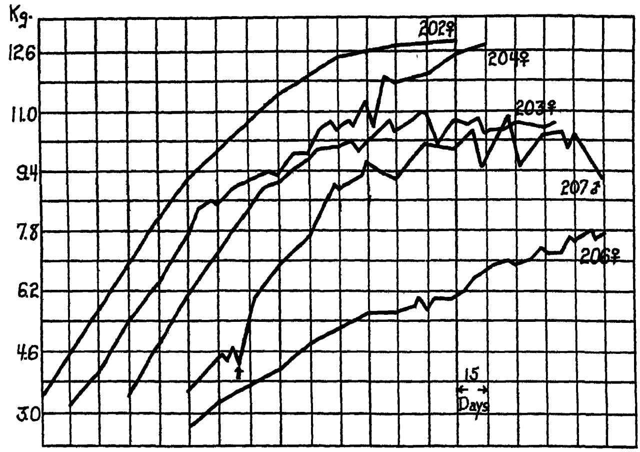

Fig. 2 Growth of five littermate puppies on the purified ration containing alcohol-extracted casein. Dogs 204, 203, 207 and 206 received basal ration with synthetic vitamins only; dog 202 received in addition, 2% liver extract powder 1: 20. ↑ = started choline 50 mg. per kilogram body weight per day.

developed a growth plateau which was corrected by the addition of liver extract to the ration as shown in table 1. Dogs 179 and 200 received all the synthetic vitamins but nicotinic acid. Anorexia with concomitant loss of weight developed which could not be corrected with nicotinic acid but was immediately corrected by supplementing the ration with liver extract as shown in table 1.

DISCUSSION

From these experiments it can be seen that no rapidly fatal deficiency appears on the purified ration supplemented with the six synthetic B vitamins, whereas in deficiency of any one

of these six, symptoms appear in young puppies in 3 to 6 weeks, in older dogs in 1 to 3 months. Deficiency of additional factors may not appear at all on the basal ration containing our acid-washed casein, whereas this ration always produces deficiency of any one of the above-mentioned six synthetic

TABLE 1

Behavior of three growing dogs receiving the basal acid-washed casein ration and various supplements.

DOG NO.	LITTER	RATION AND SUPPLEMENTS	INITIAL WEIGHT	TEST PERIOD	TOTAL GROWTH	REMARKS
			kg.	*days*	*kg.*	
172	G	Basal acid-washed casein ration	4.5	152	3.0	Good growth followed by a plateau for 57 days
172		3% liver extract powder 1: 20 (The Wilson Laboratories)	7.5	26	1.15	Excellent growth
179	H	Basal acid-washed casein; received no nicotinic acid	4.2	30	—0.5	Decline in weight
179		18.4 mg. nicotinic acid	3.7	4	+0.5	Rapid growth
179		Basal	4.2	26	—0.4	Decline in weight
179		20 mg. nicotinic acid	3.8	15	—0.15	Decline in weight
179		15.4 mg. nicotinic acid	3.65	4	0	No response
179		10 gm. 1: 20 liver extract powder	3.65	16	+0.75	Excellent growth
200	I	Basal acid-washed casein ration; received no nicotinic acid	1.7	12	+0.05	Poor growth
200		10 mg. nicotinic acid	1.75	11	0.10	No response
200		30 mg. nicotinic acid	1.85	9	—0.05	No response
200		2% liver fraction "D" (The Wilson Laboratories)	1.75	40	2.15	Good growth

vitamins. However, if the acid-washed casein is purified further by alcohol extraction, deficiency characterized by anorexia and loss of weight usually appears after 1 to 3 months. In several dogs we have observed a slight to marked greying of the hair. From our studies on pantothenic acid

deficiency in dogs (unpublished data) we feel fairly certain that the possibility of this greying being due to pantothenic acid deficiency may be ruled out.

There seems to be, therefore, a qualitative difference between the apparent requirement of the dog for thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, and choline, and that of the other factors in the vitamin B complex when one compares the speed of onset and the acuteness of the deficiency of the first six members with that of the other factor in liver extract. The dietary requirement for the liver factor may be lessened by the ability of the animal to effect a partial synthesis in the tissues or in the intestine by bacteria.

If intestinal synthesis of this factor accounts for the differences observed in apparent requirement between this factor and the other members of the vitamin B complex, any dietary changes which might affect the intestinal flora might be expected to interfere with the synthetic processes. The results obtained with dogs 179 and 200 receiving no nicotinic acid might be explained on the basis that dietary nicotinic acid was essential in some manner for the bacteria responsible for the synthesis of the liver factor. When nicotinic acid was removed from the diet synthesis of the liver factor was curtailed and the dogs would no longer respond to nicotinic acid but would respond to liver extract. The addition of certain bacteriostatic drugs such as sulfaguanidine to the ration might also interfere with these syntheses as suggested by experiments with rats (Black, McKibbin and Elvehjem, '41). Such an approach might throw considerable light on this question.

The identity of the substance or substances in liver extract responsible for the improvements recounted above cannot be established from these studies. Inositol has been shown to be essential in the nutrition of yeast (Koser and Saunders, '38) and mice (Woolley, '40). The role of inositol in rat and chick nutrition is less clear. From our results it seems apparent that inositol alone at levels comparable to those found effective in other species does not replace liver extract.

The suggested role of p-amino benzoic acid in the nutrition of the rat and chick (Ansbacher, '41) as well as its capacity to neutralize the bacteriostatic action of sulfanilamide on certain microorganisms suggests the possibility of its importance in dog nutrition. The use of glutamine was suggested by its growth-stimulating action on certain bacteria (Feeney and Strong, in press). Preliminary evidence indicates that these substances will not replace liver extract when given in combination with inositol. All these substances, however, may deserve further consideration as essential elements in dog nutrition and will be checked further.

The possible role of biotin in the nutrition of the dog is still obscure. We have not as yet substituted biotin concentrates for liver extract in these studies.

Studies on pantothenic acid and nicotinic acid deficiencies on this acid-washed casein ration supplemented only with synthetic vitamins are now in progress.

SUMMARY

1. Dogs receiving a highly purified vitamin B complex free ration supplemented with thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, and choline may grow erratically and suboptimally. Growth may be increased by the addition of liver extract to the ration.

2. By further purification of the casein in this ration growth is usually arrested and anorexia and loss of weight occur. This condition is curable by liver extract. Preliminary evidence indicates that the condition is not curable by mixtures of inositol, p-amino benzoic acid, and glutamine.

3. The onset of this deficiency is much slower and more chronic than is observed with those six B vitamins mentioned above.

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AVAILABILITY TO RATS OF PHOSPHORUS IN RED CLOVER HAYS OF WIDELY VARYING PHOSPHORUS CONTENT

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THE PROBLEM

Previous work with rats (Williams, MacLeod and Morrell, '40 a, b) indicated that animals fed a diet containing an amount of phosphorus minimal for growth, one-half of which came from a low phosphorus hay, gained less weight and stored less phosphorus over a 30-day experimental period than animals fed a diet containing the same amount of phosphorus, one-half of which came from a high phosphorus hay of the same type. Similar results were obtained whether the hays used were lespedeza sericea, alfalfa, soybean hay, or red clover. This work has now been extended to show the effect on white rats of the addition of (1) a liberal phosphorus supplement to each ration containing the hays of differing phosphorus content, and (2) the ash of the two hays to a stock ration in amounts equivalent to the hay in the preceding rations. The first procedure was adopted in an attempt to determine whether or not the mere addition of a phosphorus supplement to the diet containing the low phosphorus hay would make it as adequate as that containing the high phosphorus hay. The purpose of adding the phosphorus of the hay in the form of its ash rather than as the whole product was to eliminate from consideration all differences in bulk and in organic materials. Previous work has noted the marked

differences in amounts of feces due to the different diets, and has recognized the possibility of differences in quality not brought out by routine analysis of the two hays, such as in the fraction designated "nitrogen-free extract." If differences in animal response still persisted when the hay was fed as ash, the explanation, in part at least, of the cause for the differences would have been narrowed down to the mineral fraction of the hays.

EXPERIMENTAL PROCEDURE

Essentially the same experimental procedure was used for these experiments as that reported in earlier articles. The red clover hays were the same as used previously, the phosphorus analyses being as follows: the low phosphorus red clover, 0.12% (0.27% P_2O_5); and the high phosphorus red clover, 0.31% (0.71% P_2O_5). Tables 1 and 2 show the composition of the experimental diets. In the diets containing the red clover hay, all essential nutrients were present in adequate amounts, including phosphorus which was at the level of

TABLE 1

Percentage composition of experimental rat diets.

COMPONENT	RED CLOVER HAY			RED CLOVER HAY ASH[1]		
	Control	Low P hay	High P hay	Control	Low P ash	High P ash
	%	%	%	%	%	%
Hay or ash		69	26		4.7	1.7
Lactalbumin[2]	18	11	15	18	18	18
Starch[2]	49	6	34	49	44.3	47.3
Agar	19	0	11	19	19	19
Salt mixture[3]	4	4	4	4	4	4
Butterfat	8	8	8	8	8	8
Cod liver oil	2	2	2	2	2	2

[1] Hay ash diets for rats in series I and II were identical except that agar was not included in the diets for series II and the starch was correspondingly increased.

[2] The same amount of yeast extract was included in comparable diets, being incorporated with the starch or lactalbumin.

[3] Osborne and Mendel salt mixture modified to give desired amounts of phosphorus and calcium, and by the addition of copper sulphate.

0.32% of the whole diet. In contrast to the diets in the previously reported studies which contained only a minimal amount of phosphorus at the level of 0.16%, this represented a liberal phosphorus supplement for each diet. In the diets containing the red clover ash, all essential nutrients were present in adequate amounts except for phosphorus which was at the

TABLE 2

Distribution of foodstuffs among the various constituents of the red clover hay and the red clover hay ash diets.

DIETARY FACTOR	RED CLOVER HAY DIETS			RED CLOVER HAY ASH DIETS [1]		
	Control	Low P hay	High P hay	Control	Low P ash	High P ash
	%	%	%	%	%	%
Protein:						
Hay	0	7	3	0	0	0
Lactalbumin	18	11	15	18	18	18
Total	18	18	18	18	18	18
"N-free extract":						
Hay	0	28	10	0	0	0
Starch	49	6	34	49	44.3	47.3
Total	49	34	44	49	44.3	47.3
Fat:						
Butterfat	8	8	8	8	8	8
Cod liver oil	2	2	2	2	2	2
Fiber:						
Hay	0	19	8	0	0	0
Agar	19	0	11	19	19	19
Total	19	19	19	19	19	19
Calcium:						
Hay	0	0.959	0.263	0	0.967	0.260
Lactalbumin	0.036	0.022	0.030	0.036	0.036	0.036
$CaCO_3$	0.887	0	0.718	0.868	0	0.686
Total	0.923	0.981	1.011	0.904	1.003	0.982
Phosphorus:						
Hay	0	0.080	0.080	0	0.079	0.076
Lactalbumin	0.027	0.017	0.023	0.027	0.027	0.027
H_3PO_4	0.280	0.218	0.206	0.132	0.066	0.058
Total	0.307	0.315	0.309	0.159	0.172	0.161
Ca/P ratios	3.0	3.1	3.3	5.7	5.8	6.1

[1] See footnote 1, table 1.

minimal level of 0.16%, one-half of the total coming from hay ash and the other half from a salt mixture. The hay was ashed[1] at first over an open flame and finished in a muffle furnace at 600°C. Two series of animals were run on the ash diets. Series I had agar in all diets in an amount to equal the fiber content of the corresponding diets in which the hay was fed. Series II had no agar in the diets and the starch content was correspondingly increased. The Ca/P ratios for the diets containing the phosphorus supplement were approximately 3:1 and for the diets containing the hay ash, they were approximately 6:1 which is the same ratio as published previously for the comparable diets in which red clover hay itself was fed rather than the ash.

The triplicate feeding method with equalized food consumptions was employed as in previously reported work. The animals were 30–31 days old when started on the experimental diets on which they were continued for 30 days. Rate of growth and phosphorus retention were again used as criteria in judging results.

RESULTS

Table 3 summarizes the average results obtained for all animals. It also includes data published earlier for rats on a similar ration using the same red clover hays in which the total phosphorus was at the level of 0.16%. It will be noted that for both males and females, the rats which received phosphorus at the 0.32% level, one-fourth of which came from a low phosphorus hay, were conspicuously less thrifty animals than either the controls or those deriving one-fourth of the phosphorus from a hay high in phosphorus content whether judged on the basis of gain in weight, body length, or the amount of phosphorus retained in the body. A statistical analysis by "Student's" method as given by Goulden ('39) reveals significant differences between the animals on the low phosphorus and high phosphorus hay diets both as regards

[1] The hay was ashed by Mr. B. W. Hatcher, Asst. Soil Chemist, Agricultural Experiment Station, University of Tennessee.

TABLE 3

Summary of average results for experimental rats in growth and phosphorus intake and retention.

EXPERIMENTAL GROUP	NO. OF CASES	WEIGHT			LENGTH OF TRUNK	TOTAL FECES DRY WEIGHT	FOOD CONSUMPTION	TOTAL P IN FOOD	P IN RAT AT 60–61 DAYS	P IN RAT AT 30–31 DAYS (ESTIMATED)	P STORED FROM FOOD	P FROM FOOD
		Initial net	Final net	Gain net								
		gm.	*gm.*	*gm.*	*cm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	%
Red clover hay (total P — 0.16%)												
Males												
Control	5	51	105	54	15.4	57	239	0.379	0.535	0.272	0.263	69
Low P	5	51	63	12	13.4	121	239	0.389	0.372	0.271	0.101	26
High P	5	52	92	40	14.6	91	241	0.387	0.492	0.274	0.218	56
Red clover hay (total P — 0.32%)												
Males												
Control	5	56	150	94	16.4	59	274	0.841	0.866	0.295	0.571	68
Low P	5	56	84	29	14.0	145	274	0.861	0.556	0.295	0.262	30
High P	5	56	133	73	15.4	95	277	0.856	0.747	0.295	0.452	53
Females												
Control	5	53	118	65	15.9	52	245	0.752	0.771	0.280	0.491	65
Low P	5	54	72	18	13.7	133	248	0.780	0.521	0.286	0.235	30
High P	5	53	107	54	15.0	86	250	0.772	0.685	0.281	0.404	52
Red clover ash (series I)[1]												
Males												
Control	5	56	79	23	14.4	40	165	0.262	0.492	0.296	0.196	75
Low P	5	56	61	5	13.2	39	157	0.270	0.386	0.296	0.091	34
High P	5	56	78	22	14.3	41	165	0.265	0.463	0.295	0.168	63
Red clover ash (series II)[1]												
Males												
Control	5	58	91	33	15.0	6	181	0.288	0.563	0.305	0.258	90
Low P	5	58	75	17	14.3	9	175	0.304	0.454	0.305	0.149	49
High P	5	58	88	30	15.0	8	180	0.303	0.518	0.306	0.211	70

[1] See footnote 1, table 1.

growth and phosphorus retentions; the probability being less than 1 in 100 that the observed differences are due to chance. If these results are compared with those reported in detail in an earlier paper with the same red clover hay without a phosphorus supplement, i.e., at the 0.16% phosphorus level (table 3), it can be seen that there is an improvement in growth and phosphorus retention of animals on diets containing 0.32% phosphorus over those on corresponding diets containing 0.16% phosphorus. However, the same differences between those receiving part of the phosphorus from the low phosphorus hay and those receiving part from the high phosphorus hay are apparent regardless of the amount of phosphorus in the diet. For male rats on the rations at the 0.16% phosphorus level, the percentages of phosphorus retained from the total phosphorus of the diet for the control, low phosphorus, and high phosphorus hay diets were, respectively, 69, 26, and 56; and for corresponding diets at the 0.32% level they were 68, 30, and 53. It seems possible that the constituents of the low phosphorus hay responsible for the impairment in utilization of phosphorus may act by combining with this element to form insoluble compounds. Elements which are known to form insoluble compounds with phosphorus (Schmidt and Greenberg, '35) and which have been found to be present in the hays used are iron, aluminum, barium, and calcium in excess. Quite a body of literature may be quoted giving evidence that the presence of excessive amounts of iron and aluminum prevents the proper utilization of phosphorus (e.g., Waltner, '27; Cox, Dodds et al., '31; Brock and Diamond, '34; Deobald and Elvehjem, '35; Jones, '38; Rehm and Winters, '40; Freeman and Freeman, '41). A spectrographic analysis [2] of the ash of the two hays indicated the presence of both iron and aluminum. Chemical analyses were subsequently made to determine the relative amounts of these substances in the two hays, using the A.O.A.C. method for plant materials in which the iron and aluminum were weighed

[2] Spectrographic analysis made in Dr. R. V. Allison's laboratory at the University of Florida.

as phosphates. Table 4 presents the experimental data obtained on the basis of these analyses. Assuming for purposes of comparison that all the phosphates were in the form of iron phosphates (calculated as Fe PO_4) and then as aluminum phosphates (calculated as Al PO_4), it was found that the phosphorus contents represented by these percentages of phosphate were from 0.06 to 0.08% for the high phosphorus hay and from 0.09 to 0.11% for the low phosphorus hay. In the high phosphorus hay there was a total phosphorus content of 0.31% and therefore, even though all the iron and aluminum present combined to form insoluble phosphates, there would

TABLE 4

Comparison of the percentages of iron and aluminum phosphates[1] *in the low phosphorus and high phosphorus hays.*

	0.12% P HAY	0.31% P HAY
Per cent Fe — and $AlPO_4$ in ash	6.23	4.33
Per cent ash in hay	7.18	6.82
Per cent Fe — and $AlPO_4$ in hay	0.447	0.295
Per cent P involved if all PO_4 is $FePO_4$	0.089	0.061
Per cent P involved if all PO_4 is $AlPO_4$	0.110	0.075
Per cent P involved if PO_4 is one-half $FePO_4$ and one-half $AlPO_4$	0.100	0.068
Per cent P left in hay if all possible combined as one-half $FePO_4$ and one-half $AlPO_4$	0.02	0.24

[1] Calculated as $FePO_4$ and $AlPO_4$ respectively.

still be a large margin of safety of phosphorus available to the animal. In the low phosphorus hay, on the contrary, with a total phosphorus content of 0.12%, a complete precipitation of phosphorus by the iron and aluminum would leave little or practically no phosphorus available to the animal. If the relationships between phosphorus content and iron and aluminum content noted in these particular hays prove to have a correlation as regards hays of varying phosphorus content grown on different soil types, we believe that a chemical explanation, or at least partial explanation, may have been found for the differences in the availability of phosphorus previously noted within the same type of forage. It is recognized that other

substances besides iron and aluminum, such as barium, fluorine, or excess calcium, may take part in this depressing effect.

This explanation is in essential agreement with that given by Shields et al. ('40) for differences in utilization of calcium in various food materials. These authors state that "the constituents of vegetables responsible for the impairment in calcium utilization act either by combining with the calcium to form insoluble compounds, as the oxalates of foods are known to do, or to modify unfavorably the reaction of the intestinal contents, or to increase intestinal motility. These factors must account for the so-called 'associative effects' of foods in digestion, whereby one food will impair the digestibility of another."

The results obtained from the rats fed the red clover hay in the form of ash give further corroborative evidence for the above interpretation. In this case the three experimental rations were nearly identical except that in the control ration all the phosphorus was supplied by a salt mixture and in the others one-half the phosphorus was supplied by a small amount of low phosphorus hay ash in one case and by a smaller amount of high phosphorus hay ash in the other. Any interfering minerals present in the ash would have a chance to combine immediately with the phosphorus from any source without elapse of time for the digestion of the organic cellular matter of the hay. This more intimate mixing of the elements in the ash may be the explanation for the drastic effect of the low phosphorus ash diet on the appetite of the rats to which it was fed. They ate barely enough to maintain their weight throughout the experimental period and thus restricted the food consumption of their litter mates in the trio. The small food consumption would seem to account for the small gain in weight for all rats. The differences in growth of the animals on the hay ash in series I and II may be related to the fact that in the first series agar was included in the ration to make the fiber content the same as when the hay itself was fed and in the second series, the agar was omitted. Again we note a

significant difference between rate of growth and phosphorus retention in the animals receiving the low phosphorus hay ash as compared with those receiving the control diet or the diet containing the high phosphorus hay ash. In the first series, the percentages of phosphorus retained from the total dietary phosphorus were comparable to those obtained when one-half the phosphorus was supplied as hay, the values being for the control, low phosphorus ash, and high phosphorus ash diets, respectively, 75, 34, and 63. In the second series the percentages for the corresponding diets were higher, namely, 90, 49, and 70, but in both series there was a marked difference between the rats fed the low phosphorus ash and those fed the control and high phosphorus ash diets. For the two series, the probability was about 1 in 100 that the observed differences were due simply to chance variations. These results seem to indicate that at least a partial explanation for the differences in the availability of phosphorus in these red clover hays of varying phosphorus content may be assigned to the mineral fraction of the hays in question. The fact that the total amounts of feces eliminated on the three diets compared were almost identical seems to rule out this factor as a causative agent.

SUMMARY OF RESULTS

1. When the experimental diets contained all nutrients including phosphorus at the same and optimal levels of adequacy, the phosphorus of a diet containing low phosphorus red clover hay was less available to the rat for growth and bone development than the phosphorus of a diet containing high phosphorus hay of the same type. Evidence is presented indicating that the amounts of iron and aluminum present in the low phosphorus hay are sufficient to be a possible disturbing factor in the utilization of the phosphorus by the formation of insoluble phosphates.

2. When the experimental diets contained phosphorus at a minimal level of adequacy and all other nutrients at adequate levels and essentially the same for all diets, the phosphorus

of a diet containing low phosphorus red clover hay supplied in the form of ash was less available to the rat for growth and bone development than the phosphorus of a ration containing high phosphorus red clover hay ash.

CONCLUSION

The present study gives some evidence for believing that the observed differences between animals receiving the low phosphorus hay and those receiving the high phosphorus hay can be explained, in part, as a difference in availability of the phosphorus in the two hays due to a difference in the ratio of the amount of phosphorus present to the amounts of substances like iron and aluminum capable of forming insoluble phosphates in the intestinal tract and thus interfering with phosphorus assimilation.

More work needs to be done in extending our knowledge of the chemical quality of feeds by the use of methods other than those now in common practice for feed analysis. This study represents only a preliminary and fragmentary method of attack. The work should be repeated with animals having digestive tracts suited to consuming large amounts of roughage, as has been pointed out by L. A. Maynard ('41) in reference to our previously published articles. Work is at present under way using an herbivorous animal; also further work with rats using different feeding regimes in an attempt to induce all the animals on the ash diets to eat more and thus to make more nearly normal gains in weight. The authors believe, however, that the objectives of the present study are in line with the recent trend in agricultural and nutritional research as expressed by E. C. Auchter ('39) when he says in connection with the problem of human nutrition, "It is a problem of crop production, of food production . . . . But hitherto we have thought of it too largely in terms of quantity . . . . It would be no revolutionary step for us to think in terms of *nutritional* quality as well."

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ALPHA-TOCOPHEROL REQUIREMENT OF THE MOUSE

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Vitamin E prevents sterility in female mice reared upon synthetic diets (Beard, '25–'26; Bryan and Mason, '40). The sterility was shown by Bryan and Mason to be characterized by death and resorption of the fetuses and to be indistinguishable from that observed in rats upon E-deficient diets (Evans and Bishop, '23; Evans and Burr, '27; Urner, '31). Bryan and Mason ('40) observed no symptoms of neuromuscular disturbance near the end of lactation in the young of females upon synthetic low-E diets, such as are frequently seen in young suckling rats under similar conditions (Evans and Burr, '28; Olcott, '38). They found that male mice on the E-deficient diet failed to develop the testicular degeneration typical of male rats that have been deprived of vitamin E (Mattill and Stone, '23; Mason, '26; Evans and Burr, '27).

This is a report upon (1) the α-tocopherol requirement of the female mouse for the prevention of resorption during gestation; (2) the occurrence of muscular dystrophy in the young of females on E-deficient diets; and (3) the failure of male mice to develop testicular degeneration even after the possibility of storage of vitamin E had been reduced to a minimum.

The pathology of these mice was investigated by Dr. A. M. Pappenheimer ('42).

EXPERIMENTAL

The diets, supplements and methods were similar to those used for rats (Goettsch and Pappenheimer, '41).

Diets and supplements. The diets (I and II), similar to those used by Evans and Burr ('27), had the following percentage composition:[1] casein[2] 29.1 (26.6); cornstarch, raw 36.3 (33.3); lard 20.0 (18.3); yeast, bakers' dried 9.0 (16.6); salt mixture (Hawk and Oser, '31) 3.6 (3.4); and cod liver oil[3] 2.0 (1.8). Diet II containing more yeast was used during the period of lactation. The diets were freshly prepared every week.

The stock diet had the following percentage composition: whole wheat 55.5; wheat germ 10.0; casein 10.0; whole milk powder 15.0; $CaCO_3$ 1.5; NaCl 1.0; cottonseed oil[4] 5.0; and cod liver oil 2.0.

Three preparations of α-tocopherol were employed: synthetic dl-α-tocopherol acetate[5] and natural and synthetic α-tocopherols.[6] The tocopherols were dissolved in sesame oil. The solutions were prepared by weight in such proportions that 0.25 mg. of α-tocopherol was contained in 1 drop, and stored at 4°C.; they were used within 1 week of preparation.

METHODS

Young female mice from a stock colony maintained on a commercial dog food[7] were reared on diet I from the time of weaning (21 days). They were weighed every fifth day on a horn pan balance. Vaginal smears were made daily. At about 60 days of age, they were bred with normal males during estrus. If litters were cast, the females were bred until the occurrence of gestational resorptions.

[1] The figures for diet I are those which immediately follow the name of the ingredient; the figures for diet II are given in parentheses.

[2] Merck's technical.

[3] Mead Johnson.

[4] Wesson.

[5] Ephynal, of Hoffman-La Roche, Inc., through the courtesy of Dr. F. Gudernatsch.

[6] Merck and Co., through the courtesy of Dr. J. M. Carlisle.

[7] Purina Chow.

Whenever a gestation resulted in resorption of fetuses, the mice were bred again and given orally, on or before the fifth day after mating, single doses of wheat germ oil or of α-tocopherol. Observations were made upon the number of litters, litter size, and young born alive, which survived the early lactation period and developed muscular dystrophy. After the period of lactation the females were bred to determine the number of litters produced by a single dose of vitamin E.

The young which survived lactation were given diet I. Growth, reproduction and the response to α-tocopherol were observed in the females as in the first generation. Their progeny became the third generation. Seven generations were reared upon diet I with sufficient vitamin E to permit the birth of living young and their survival.

Some males and females of the third generation were reared on the stock diet containing wheat germ and cottonseed oil [8] as sources of vitamin E. Female offspring, reported as the first generation, were reared on diet I and their fertility recorded.

Procedure during gestation. It was more difficult to determine the occurrence of positive mating in the mouse than in the rat (Evans and Burr, '27), for in the mouse the plug apparently does not remain in place so long, nor are sperm often found in smears after the plug has dropped out. By examining the mated mice several times during the day it was possible to observe the plug in about two-thirds of the positive matings; in the other third, pregnancy was determined by the absence of estrus and the finding of the "placental sign" in the daily smears. In all pregnancies, implantation was indicated by the presence of erythrocytes in the smear on the twelfth day after mating. Failure of implantation after positive mating occurred in 32 (7%) of 444 gestations on diet I.

Litters were cast on the twentieth day of pregnancy. In resorption gestations there was a gradual loss of 2 to 8 gm. in body weight from the eighteenth to twenty-second day.

[8] See footnote 4, page 514.

Procedure during lactation. Whenever there were living young, the female was given diet II and permitted to suckle them. Record was made of the number of young which survived the first 10 days of lactation, and during the rest of the period the mice were observed daily for symptoms of muscular dystrophy. Any mice which appeared to be abnormal were killed, autopsied, and specimens preserved for histological examination. After the period of lactation the females were given diet I, and bred again.

At the termination of the experiments, forty females and their young were killed during different stages of lactation and examined.

Males. Males of the second to the seventh generations were reared on diet I. Some were given in addition, 1 or 2 drops of wheat germ oil daily or 1.0 mg. of α-tocopherol per week. They were weighed every fifth day and bred after the sixtieth day with normal females in estrus. Some were under observation during their life span, which rarely exceeded 400 days. At autopsy of males that had been killed, smears from the vas deferens in Locke's solution were examined for motility of sperm, and specimens of testes, muscle, and occasionally central nervous system were preserved.

As soon as it became apparent that the males never developed testicular degeneration under these conditions, males and females were reared together on diet I and not separated until the females were pregnant. The age at which males matured was thus determined.

RESULTS

Growth and estrus in female mice on diet I. Growth, age at the time of first estrus, and the length of estrus cycles of females of all generations reared on diet I were similar to those of controls on the stock diet. The mean body weight of thirty-five females at 60 days of age was 25.0 ± 3.4 gm. The first estrus occurred at 32.6 ± 4.6 days at a body weight of 18.1 ± 2.5 gm. The first estrus cycle varied between 6 and 14 days in length, but thereafter the cycles were regular and

of 4 to 6 days' duration (Allen, '22). In 132 cycles, the mean length was 5.5 ± 1.7 days. These findings are in accord with those observed in female rats on synthetic E-low diets (Evans and Bishop, '23).

Fertility on diet I. As proposed by Bacharach and Allchorne ('38), this was expressed as the per cent of gestations which resulted in the birth of young, regardless of litter size or number of living young.

The fertility of female mice that were reared on diet I from the time of weaning (twenty-first day) is shown in table 1. It will be seen that the fertility varied from 0 to 62%.

TABLE 1

Fertility of female mice on vitamin E-low diet I; showing effect of maternal diet on fertility of offspring.

NUMBER OF GENERATION	NUMBER OF			PER CENT FERTILITY	DIET OF MOTHER DURING LACTATION	SINGLE DOSE OF α-TOCOPHEROL GIVEN MOTHER AT BEGINNING OF GESTATION
	Mice	Gestations	Litters			
						mg.
1	15	13	0	0	Purina Chow	0
	13	13	8	62	Stock diet	0
2	23	22	0	0	Diet II	*
	14	14	2	14	Diet II	0.5
	12	12	5	42	Diet II	1.0
3	44	42	4	10	Diet II	0.5
4	20	20	0	0	Diet II	1.0
5	8	8	0	0	Diet II	1.0
	9	8	0	0	Diet II	2.0
6	6	6	3	50	Diet II	1.0
7	8	7	1	14	Diet II	1.0

* Mother given 25 drops of wheat germ oil.

Since the fertility appeared to be influenced by the amount of vitamin E which was obtained and stored by the young during gestation and lactation (Mason and Bryan, '38), data on the diet of the mother and the amount of α-tocopherol given to her in a single dose at the beginning of gestation were included in the table. When the females with litters were subsequently bred there were only two second litters, both in the group

in which the mothers had been fed the stock diet. From table 1 it is evident that the fertility of sixth and seventh generation mice did not differ appreciably from that of the second.

Curative effect of α-tocopherol. The response of sterile female mice on diet I to single doses of α-tocopherol at the beginning of gestation is shown in table 2. Since there were

TABLE 2

Prevention by α-tocopherol of resorption during gestation in mice of proved sterility on vitamin E-low diet I.

AGE OF MOUSE	α-TOCOPHEROL: SINGLE DOSE AT THE BEGINNING OF GESTATION		NUMBER OF		PER CENT FERTILITY
	Kind	Dose mg.	Gestations	Litters	
3–6 months	*Natural*	0.25	13	8	62
		0.50	10	9	90
3–6 months	*Synthetic*	0.25	6	3	50
		0.50	11	8	73
		1.00	6	5	83
		2.00	4	4	100
3–6 months	*Synthetic acetate*	0.25	12	6	50
		0.50	41	33	80
		1.00	20	18	90
7–12 months	*Synthetic acetate*	1.00	12	6	50
		2.00	12	7	58
		5.00	19	18	95

no apparent differences among them, the data obtained from the seven generations of mice were combined in this and the following tables. It will be seen that, as in the rat (Goettsch and Pappenheimer, '41), the three preparations of α-tocopherol gave essentially the same results: 0.25 mg. induced litters in seventeen (55%) of thirty-one mice; 0.50 mg., in fifty (80%) of sixty-two mice; at 1.0 to 2.0 mg., the incidence was higher. As in the rat, the biological variation was very great. In older mice the requirement for vitamin E was greater, as has been reported for the rat (Emerson and Evans, '39).

The minimum amount of α-tocopherol which permitted the birth of young to at least 85% of the mice was approximately 0.5 to 1.0 mg. under the given experimental conditions. This

value is higher than that reported for the mouse by Bryan and Mason ('40) and is roughly one-third of the amount, 1 to 3 mg., required by the rat for the birth of living young (Evans, Emerson and Emerson, '36; Karrer and Keller, '38; Bacharach, '38; Goettsch and Pappenheimer, '41).

Calculated by the surface area formula of Meeh and the constants of Rubner (Lusk, '28), the body surface of a 200-gm. rat is 311.2 cm². ($9.1 \times 200^{2/3}$), and that of a 25-gm. mouse, 97.5 cm². ($11.4 \times 25^{2/3}$). The body surface of the mouse is therefore about one-third as great as that of the rat. These data suggest that per body surface area, the α-tocopherol requirement for the prevention of resorption during gestation is the same in the rat and mouse.

Second litters were rarely born after single doses of α-tocopherol, and there were no third litters.

Litter size. The litters varied in size from one to fourteen mice, averaging 6.7 gm. for the controls on the stock diet and for mice on diet I that were fed 1.0 mg. α-tocopherol. It will be seen in table 3 that litters tended to be smaller both

TABLE 3

Influence of α-tocopherol upon litter size, the number of young born alive, surviving the early lactation period, and developing muscular dystrophy.

	MG. OF α-TOCOPHEROL						STOCK DIET B
	Single dose at beginning of gestation						
	0 [1]	0.25	0.50	1.0	2.0	5.0 [2]	
No. of litters	25	17	50	29	10	18	10
No. of young	125	104	309	195	56	95	67
Av. young per litter	5.0	6.1	6.2	6.7	5.6	5.3	6.7
No. living young	110	93	272	180	45	88	65
Per cent	88	89	88	92	80	93	97
No. surviving 10 days	64	68	207	126	40	50	63
Per cent	58	73	76	70	89	57	97
No. with muscular dystrophy	7	8	27	20	4	8	0
Per cent of those surviving	11	12	13	16	10	16	0

[1] First litter fertility.

[2] Older animals.

in older mice and in those that received lesser amounts of vitamin E. The average birth weight was 1.34 gm. for 181 mice of the fourth and fifth generations.

Number of living young. It will be seen in table 3 that in all litters on diet I, 80% or more of the young were living at the time that the litters were found, regardless of the amount of α-tocopherol.

The cause of death in the newborn mice was undetermined but lesions of muscular dystrophy were found in thirteen of twenty-two that were examined histologically.

Survival of young during lactation. As shown in table 3, 70 to 89% of young mice born to females on diet I with 0.25 to 2.0 mg. of α-tocopherol survived the first 10 days of lactation. The young of older females and those born as a result of "first litter fertility" did not survive so well.

Of sixty-three mice that did not survive and were examined histologically, ten presented typical lesions of muscular dystrophy.

Muscular dystrophy. Although the young mice were observed daily during lactation for symptoms of "paralysis" such as occur under similar circumstances in the rat (Evans and Burr, '28), these never appeared (Bryan and Mason, '40). A few mice died, some developed queer darting movements, and some became emaciated. At autopsy, the muscle seldom appeared abnormal, but occasionally it had a cheesy consistence or was streaked with yellowish grey. Microscopically there was hyaline necrosis of the muscle fibers with early calcification and active regeneration. Muscle lesions were occasionally found in older mice. There were no other changes of significance in either the brain, cord, peripheral nerves, bones, nor in any of the viscera examined (Pappenheimer, '42).

Since the mice presented no symptoms it was impossible to tell how many of the young of the second to the seventh generations had the disease during lactation and spontaneously recovered. The incidence of muscle disorder, given in table 3, might have been greater if all of the young had been killed and

examined. The disease occurred sporadically in about 15% of the young, rarely affecting all members of a litter. In the rat under similar conditions muscular dystrophy occurs in 90% of the young from the seventeenth to twenty-third day of lactation, with a mortality of 35%, and is prevented by the administration of 0.5 mg. of α-tocopherol on the fifteenth day (Goettsch and Pappenheimer, '41). The preventive effect of α-tocopherol in mice was not investigated.

Growth of male mice on diet I. Growth of males of all generations reared on diet I was similar to that of controls on the stock diet. The mean body weight at 60 days of age of fifteen males on diet I was 29.0 ± 2.9 gm. In twelve littermates that were given wheat germ oil or α-tocopherol the mean body weight at 60 days was 28.7 ± 3.0 gm. Subsequently there was no difference in the rates of growth between the various groups.

With respect to growth, male mice respond differently from male rats to synthetic E-low diets, for in the latter, growth is invariably retarded and is restored by vitamin E (Evans and Burr, '27).

TABLE 4

Fertility of male mice on vitamin E-low diet I.

	GENERATION					
	2	3	4	5	6	7
Number of mice	21	21	14	5	3	3
Number siring litters	21	21	14	5	3	3
First fertile mating						
Number of mice	6	8		5	3	3
Age in days, av.	60	66		62	58	45
Age in days, extremes	(40–81)	(55–81)		(55–75)	(36–76)	(39–59)
Last fertile mating						
Number of mice	13	11	7			
Age in days, av.	190	216	182			
Age in days, extremes	(128–299)	(144–376)	(105–255)			
Age at time of death						
Number of mice	13	11	7			
Days, av.	298	283	283			
Days, extremes	(154–341)	(166–486)	(169–392)			

Fertility of males on diet I. As will be seen in table 4, all of the males of the second to the seventh generations were fertile. These results, though in complete accord with those of Bryan and Mason ('40) are surprising inasmuch as male rats of the second and third generations are invariably sterile from the beginning of sexual maturity (Evans and Burr, '27).

Sexual maturity, as judged by the ability to sire litters, occurred at an average age of 60 days. The length of the fertile period varied greatly but did not appear to be shorter in the males on diet I than in those given vitamin E. Towards the end of the breeding period, males occasionally appeared to be sterile, as indicated by the finding of plug without implantation. However, at the time of death, both motile sperm in the vas deferens and histologically normal testes were invariably found.

SUMMARY

1. Seven generations of mice were reared on a synthetic E-low diet with just sufficient α-tocopherol fed to the females to permit the birth of living young and their survival.

2. In such mice there is neither retardation in growth nor any change in the age when the first estrus appears and in the length of the estrus cycle. Females are sterile when α-tocopherol is withheld.

3. Under the conditions described, the administration of 0.5 to 1.0 mg. of α-tocopherol at the beginning of gestation results in the birth of young to at least 85% of the females.

4. Lesions without symptoms of muscular dystrophy may occur at any age in the mouse; occasionally in the newborn and in adults, and to an extent of 15% in the young during the first 35 days of life.

5. Males retain their fertility and never develop testicular degeneration.

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CERTAIN RELATIONSHIPS OF AVITAMINOSIS A TO VITAMIN C IN THE YOUNG BOVINE [1]

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The decrease in ascorbic acid content of tissues in the vitamin A deficient dairy calf has been shown by Phillips et al. ('38) and King ('40). Sure, Theis and Harrelson ('39) have demonstrated a decreased ascorbic acid content of the tissues and blood plasma of the vitamin A deficient rat. The investigations reported here were undertaken to study the relationship between vitamin C and the characteristic effects of vitamin A deficiency as described by Walker ('36), King ('40), Moore et al. ('39, '40) and Phillips and co-workers ('38). It was also desired to study the relationship of the blood levels of vitamins A and C, and the effects of avitaminosis A on urinary vitamin C excretion.

EXPERIMENTAL PART

The data reported were taken from observations made on eleven calves, eight Holsteins and three Guernseys, which were maintained on the basal vitamin A deficient ration (Walker, '36) for 5 to 8 months. They were fed either the basal ration alone, or the basal ration supplemented by either crystalline carotene in cottonseed oil, or vitamin A as shark liver oil. Carotene and vitamin A were fed in amounts equivalent to 0–100 μg. and 0–18 μg., respectively, per kilogram of body

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weight per day. The levels fed were such as to completely prevent or produce varying degrees of vitamin A deficiency.

Weekly analyses of blood plasma for vitamins A and C, and carotene, were made by methods previously used in this laboratory (Phillips, Lundquist and Boyer, '41). Ophthalmoscopic observations for papillary edema, measurements of intracranial pressure, and determinations of the vitamin C content of the cerebrospinal fluid were made at frequent intervals on several of the calves. Intracranial pressure measurements were made by use of a water manometer, a puncture being made at the occipito-atlanto joint following the technique developed by Moore and Sykes ('41).

When increased pressure and papilledema developed, vitamin C was given to several of the calves to see if any remission of these conditions could be obtained.

In these experiments the blood plasma vitamin A values of the calves not receiving vitamin A supplements decreased to a low level of 0.03–0.05 µg. per cubic centimeter after 2 weeks on experiment. It was interesting to note that definite papilledema did not develop until after an average of 5 weeks on experiment, and a severe deficiency associated with increased intracranial pressure did not occur until an average of 8 weeks after low levels of blood vitamin A were observed. Thus the drop in blood plasma vitamin A content precedes by several weeks the development of these pathological conditions. Supplementation with subminimal amounts of vitamin A or carotene delayed the onset of the deficiency, but again low levels of plasma vitamin A were reached several weeks before marked deficiency symptoms developed. Grouping of the vitamin C analyses according to the vitamin A content of the blood plasma (table 1) showed that a definite relationship existed between the amounts of vitamins A and C present. The differences seem to be somewhat more pronounced with the Guernsey than with the Holstein breed.

Measurements of the ascorbic acid content of the cerebrospinal fluid showed that it normally contained from five to ten times as much ascorbic acid as the blood plasma. A striking

relation was found to exist between the ascorbic acid content of the cerebrospinal fluid and the degree of increased intracranial pressure (table 2). The drop in the vitamin C content of the cerebrospinal fluid with the increased intracranial pressure was much more pronounced than the corresponding decrease in the plasma ascorbic acid.

TABLE 1

Relation between blood levels of vitamins A and C.

PLASMA VITAMIN A	PLASMA VITAMIN C	
	Holstein calves	Guernsey calves
µg./cc.	*mg.%*	*mg.%*
0.02–0.05	0.207 (64)[1]	0.202 (17)[1]
0.06–0.10	0.254 (54)	0.254 (36)
0.11–0.18	0.272 (23)	0.304 (12)

[1] Figures in parentheses indicate number of analyses.

TABLE 2

Relation of intracranial pressure to vitamin C content of the cerebrospinal fluid and blood plasma.

INTRACRANIAL PRESSURE	AVERAGE PAPILLARY EDEMA	VITAMIN C CONTENT			
		Cerebrospinal fluid		Blood plasma	
		Average	Range	Average	Range
mm. H_2O	*diopters*	*mg.%*	*mg.%*	*mg.%*	*mg.%*
100–200 (normal)	0	2.30	1.64–2.42 (9)[1]	0.24	0.15–0.36 (15)[1]
200–300 (increased)	2.0	1.43	0.64–1.94 (21)	0.22	0.12–0.35 (24)
Over 300 (markedly increased)	2.6	1.28	0.64–1.84 (8)	0.18	0.10–0.29 (12)

[1] Figures in parentheses indicate number of analyses.

Several experiments were made in an attempt to ascertain if the administration of vitamin C would bring about a lowering of the intracranial pressure. Four calves were given from 5 to 6 gm. of vitamin C over a period of a week after they had developed an average elevated pressure of 310 mm. of water. In three cases the vitamin C administration was followed by a drop in pressure to an average of 180 mm. of water. In the

fourth case the pressure was not significantly lowered. Administration of ascorbic acid for periods longer than 1 week was impractical because of the moribund condition of the calves. The vitamin C content of the cerebrospinal fluid increased from 0.64 and 0.99 mg. per cent to 1.50 and 1.66 mg. per cent, respectively, in two calves in which pressure decreased during ascorbic acid administration. The calf which showed no diminution of cerebrospinal pressure had an increase in the vitamin C content of the cerebrospinal fluid from 1.63 to 2.01 mg. per cent during the period of ascorbic acid administration.

A further experiment was conducted by allowing one of the calves which had responded to vitamin C administration to again develop an increased intracranial pressure. When the pressure had reached 480 mm. of H_2O, 16 gm. of vitamin C was given over a 12-day period. No decrease in pressure occurred, although the vitamin C content of the cerebrospinal fluid was brought back to a normal value of 2.40 mg. per cent. The calf was in an extremely deficient state. Subsequent administration of large amounts of shark liver oil reduced the pressure to 300 mm. after 10 days and 170 mm. after 20 days and maintained the vitamin C content of the cerebrospinal fluid.

The decreased vitamin C in the blood and tissues found in the vitamin A deficient animal may have been due to either decreased synthesis or increased excretion of the vitamin. To ascertain if there was a decreased excretion of ascorbic acid in a vitamin A deficiency, which would indicate a decreased rate of synthesis, twenty-four rats of 30 days of age were placed on a diet low in vitamin A. The rats were fed the basal diet having a percentage composition of dextrin 68, alcohol extracted casein 18, brewer's yeast 7.9, irradiated yeast 0.1, salts 4, and corn oil 2. Twelve rats received the diet unsupplemented and twelve received in addition 1 drop of shark liver oil containing 15,000 I.U. per gram twice weekly. All rats received 2–3 mg. of α-tocopherol twice weekly. After the growth of the vitamin A deficient rats had reached a

plateau or begun to decrease, the animals were placed in metabolism cages, and urinary vitamin C determined on the daily excretions collected over a 2-week period. The results showed a marked decrease in vitamin C excretion by the vitamin A deficient rats. Deficient male rats excreted an average of 0.19 mg. of vitamin C per kilogram of body weight as compared to 0.49 mg. per kilogram for control males. Deficient females excreted 0.16 and control females 0.52 mg. per kilogram of body weight, respectively.

DISCUSSION

The striking relationship found to exist between the decreased vitamin C content of the cerebrospinal fluid and the increased intracranial pressure suggests that the low vitamin C is in some way associated with the increased pressure. The lowering of pressure in three calves following vitamin C administration lends support to this suggestion, but the failure to secure this in the fourth calf, and in the calf in which increased pressure was permitted to recur makes it impossible to assign a specific role to vitamin C in this respect. King ('40) has also suggested that vitamin C may be involved in the pathological changes characteristic of vitamin A deficiency. In relation to the cause of the increased intracranial pressure, Moore and Sykes ('41) found no abnormality in various blood and urine analyses, and no pathological changes of the choroidal plexus, arachnoid villi, and colloidal blood plasma osmotic pressure. The decreased vitamin C of the cerebrospinal fluid might be related to an increase in the volume of cranial contents postulated as a possible cause of the increased pressure (Moore and Sykes, '41); the increased volume of cranial contents being due to an actual volume increase, or to a relative overgrowth of the nervous system as indicated by Wolbach and Bessey ('40). The results of these experiments suggest that further studies should be made on the effects of ascorbic acid on the bone anomalies and other tissue changes characteristic of avitaminosis A that occur in rats (Wolbach and Bessey, '40), dogs (Mellanby, '41) and calves.

It is of interest to note that the development of avitaminosis A symptomatology and pathology does not occur until the plasma vitamin A has dropped to the low level of 0.05–0.07 μg. per cubic centimeter, or less, and remained there for several weeks. Although development of vitamin A pathology is related to the blood level of the vitamin, chronic low blood plasma levels may exist for some time before marked pathology develops.

The pronounced decrease in urinary excretion of vitamin C by the vitamin A deficient rat indicates that the decrease in the blood and tissue vitamin C of the rat, and in all probability of the cow, results not from increased excretion, due to possible kidney damage, but from decreased synthesis of this vitamin within the animal body. This decreased synthesis of vitamin C in vitamin A deficiency is of particular interest in view of the results obtained by the treatment of bovine sterility with ascorbic acid (Phillips et al., '41).

SUMMARY

Various degrees of vitamin A deficiency have been produced in the dairy calf, and the relationship of the deficiency to vitamin C studied. Urinary excretion of vitamin C by the vitamin A deficient rat has also been determined. These studies have shown:

1. The level of blood plasma ascorbic acid in the dairy calf is contingent upon the level of vitamin A, particularly so when the vitamin A values fall below 0.10 μg. per cubic centimeter.

2. The development of vitamin A pathology occurs only after the blood plasma levels of vitamin A have decreased to a low level of 0.05 to 0.07 μg. per cubic centimeter or less and remained there for several weeks.

3. The increased intracranial pressure observed in the calf suffering from vitamin A deficiency is paralleled by a marked decrease in the ascorbic acid content of the cerebrospinal fluid. The vitamin C content of the cerebrospinal fluid of the cow is normally five to ten times that of the blood plasma.

The administration of vitamin C to A-deficient calves was accompanied by a rise in the vitamin C content of the cerebrospinal fluid, a reduction of the cerebrospinal pressure occurring in three out of five cases. It appears that vitamin C is in some way associated with the increased intracranial pressure.

4. The urinary excretion of vitamin C in the vitamin A deficient rat is greatly reduced, thus indicating that the lowered blood and tissue vitamin C is the result of impaired synthesis.

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ADEQUACY OF SIMPLIFIED DIETS FOR GUINEA PIGS AND RABBITS [1]

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TWO FIGURES

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It was announced some years ago (Hogan and Ritchie, '34) that rabbits may be reared, with a normal rate of growth, on simplified rations. A few preliminary trials with guinea pigs were also described and it was stated that except for vitamin C, the nutritional requirements of these two species are identical. We have continued ever since to rear both species on simplified rations, with no nutritional failures. There is no doubt that our first success was obtained by adding 1% of wheat germ oil to the inadequate lard-containing basal diet. It was impossible at the time to decide whether the essential nutrient carried by wheat germ oil was vitamin E, but the identity was established as soon as alpha tocopherol was available. The requirement of the rabbit for alpha tocopherol has been demonstrated by Goettsch and Ritzmann ('39) and by Mackenzie and McCollum ('40).

Madsen, McCay and Maynard ('35) used a basal synthetic diet very similar to ours, except that it did not contain wheat germ oil, and the amount of yeast, 5%, was probably below the optimum level. Both the guinea pigs and rabbits developed degeneration of the skeletal muscles and died. When the cod liver oil was replaced by a vitamin A–D concentrate the appearance of muscle lesions was greatly delayed, but unless the

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ration was changed, the animals finally succumbed. According to our experience the provision of wheat germ oil would have permitted both the guinea pigs and rabbits to survive. Madsen ('36) observed that cottonseed oil has some protective effect against muscular dystrophy, but the protection was not complete.

Kohler, Elvehjem and Hart ('38) reported that guinea pigs declined in weight and died when supplied with a diet of mineralized milk. Supplements of either dried pork liver, a liver extract, or dried yeast, did not make the ration more adequate. Barley and wheat grass were quite potent as supplements to the milk diet; oat grass was less potent. Twenty cubic centimeters daily of the juice expressed from fresh lawn clippings was quite effective and this was interpreted to mean that this supplement contains liberal amounts of a vitamin that is absent from yeast and from liver extracts, or is present in them in only negligible amounts. This new vitamin was designated by the authors as the "grass juice factor." Since we have not used the diet of Kohler, Elvehjem and Hart we are unable to comment on their observations. As will appear later, however, our experience does not indicate the existence of a new vitamin, if its absence from yeast or from liver extracts is the criterion. There has never been any reason to doubt that these supplements contain vitamins which have not yet been recognized, but according to our interpretation it was not intended to identify these with the "grass juice factor."

Cannon and Emerson ('39) reported that guinea pigs fail to grow on their simplified rations. As an example diet 804 is described herewith, together with its percentage composition: Casein 24; sucrose 50; lard 14; agar 4; salts 4; cod liver oil 2; wheat germ oil 2; yeast extract [2].

The failure of guinea pigs to grow on the diet of Cannon and Emerson cannot be explained by the information now available to us. The diet contains 2% of wheat germ oil which our experience indicates is ample. The water soluble vitamins

[2] Equivalent to 10 parts of brewers' yeast.

are supplied by a water extract of yeast, and according to our experience it should be satisfactory. In their ration the carbohydrate component was supplied as sucrose; in ours as dextrinized corn starch; but according to our experience with sucrose rations this difference is of no significance. When either lettuce, young timothy grass, or an aqueous extract of the grass was included in the ration of Cannon and Emerson the animals were normal in appearance and in rate of growth. An aqueous extract of pork liver was ineffective. Cannon and Emerson interpreted their data as a confirmation of the existence of a grass juice factor, as announced by Kohler, Elvehjem and Hart ('38).

Randle, Sober and Kohler ('40) reported that a commercial liver extract they studied had little activity. Clark ('41) also reported nutritional failure in guinea pigs reared on a simplified ration, unless the diet was fortified by a pulverized dehydrated grass. The water-soluble vitamins of the basal diet were supplied by 3% of a commercial yeast concentrate and 0.1% of ascorbic acid, but it was not demonstrated that the concentrate was equivalent in activity to the yeast from which it was prepared.

EXPERIMENTAL

The rabbits, or Belgian hares, were reared in the laboratory and weaned at 4 weeks when they weighed approximately 450 gm. The guinea pigs, also reared in the laboratory, were weaned at 4 weeks, or if unusually large, when they weighed approximately 250 gm. The animals were placed on the experimental rations at the time they were weaned. These animals do not consume food readily if it differs in appearance or texture from the diet to which they are accustomed, and rabbits especially must be taught to consume simplified diets. It saves time if the animals are trained before weaning to consume diets similar to those they are to receive afterwards. If they have not been properly trained the experimental diet may be mixed with gradually decreasing proportions of

natural foodstuffs and in this way they learn to consume the experimental diets in a week or less.

The experimental diets may be divided into three types. The first contains dried yeast, or a yeast extract. The second type, which has not been entirely adequate, contains pure vitamins only and no crude vitamin carriers. The third contains the pure vitamins, fortified with various liver fractions. A considerable number of modifications of these rations have been prepared, but for all practical purposes the first and third types are equally adequate for growth. Space does not permit a complete description of all rations but a few typical examples are described in table 1. A few of the animals mentioned actually received a slight modification of the diet ascribed to it, but the differences proved to be of no consequence and will be ignored. In addition to the ration described, each guinea pig received a separate supplement of vitamin C. The amounts ranged from 1 to 20 mg. daily, but in our experience 5 mg. is enough for optimum growth. Dann and Cowgill ('35) state that the guinea pig requires from 0.6 to 0.7 mg. ascorbic acid for each 100 gm. body weight.

In all, approximately 125 guinea pigs and 35 rabbits have been reared to maturity, or nearly so, on simplified rations. The numerous modifications are due largely to the fact that the diets were used primarily for studies of nutritional requirements during gestation and lactation, and were changed in efforts to improve them. Our studies of this topic have not been completed and a full report will be made in a subsequent publication. For the present it will suffice to say that up to date both rabbits and guinea pigs have reared litters successfully when the diet contained vitamins E and K. Both species have made normal growth without added vitamin K. A few examples of success in reproduction will be brought out incidentally later. Since these rations were prepared primarily for lactating animals the amount of yeast was usually kept at a high level of 15%.

The response of guinea pigs to typical rations is shown in figure 1. On rations 4163, 5162, 5475 and 5096 the animals were

TABLE 1

Experimental diets that contain crude vitamin carriers.

RATION NO.	4163	5162	5097	5098	4504	5475	4180	5096
	gm.	*gm*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Casein	20	20	20	20	20	20	20	20
Dextrin	35	35	45	40	40	40	33	40
Cellulose	15	15	15	15	15	15	15	15
Lard		10	10	10		10	7	10
Soybean oil	10				10		8	
Salts	4	4	4	4	4	4	4	4
Yeast	15	15	5	10				
Yeast extract					10	10		
A–D Mixture	1	1	1	1	1	1	1	1
Tikitiki (Wells, '21)							2	
Extract 4303							4	4
Extract 4080							6	6
						mg.		
Alpha-tocopherol [1]		5	5	5		4		4
Synthetic vitamin K [1]		5				3		2
Water-soluble vitamins [1]							Footnote [2]	Footnote [3]

Notes on constituents of the rations.

Casein: Thoroughly extracted with dilute acetic acid, dried and ground.

Dextrin and cellulose: Two parts of starch to 1 part of cellophane were made into a thick paste, heated in a boiling water bath for 1 hour, dried, and ground.

Salts: 97.5% of the mixture of Hubbell, Mendel and Wakeman ('37) and 2.5% of manganous sulphate, $MnSO_4$, $4H_2O$.

Yeast: Northwestern, or Anheuser-Busch dried brewers' yeast.

Water extract of yeast: Baker's yeast was extracted with boiling water. The extract was clarified in a Sharples supercentrifuge, and concentrated in vacuo to a paste.

Vitamin A–D mixture: Lard 98, Percomorph liver oil (generously supplied by Mead, Johnson and Co., Evansville, Ind.) 2 (12,000 I. U. vitamin A, 1700 I. U. vitamin D per kilogram diet).

Liver extract 4303: Hot alcohol extract of dried beef liver. Water soluble portion, concentrated to a paste.

Liver extract 4080: The liver residue from extract 4303 was extracted with boiling water, and the extract concentrated to a paste. Additional details of the preparation of these extracts have been published by Hogan, Richardson, Patrick and Kempster ('41).

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[1] Generously supplied by Merck and Co., Rahway, N. J.

[2] Thiamine 0.2 mg., riboflavin 0.4 mg.

[3] Thiamine 0.2 mg., riboflavin 0.4 mg., pyridoxine 0.4 mg., Ca-pantothenate 2.0 mg., nicotinic acid 2.0 mg., choline 100 mg.

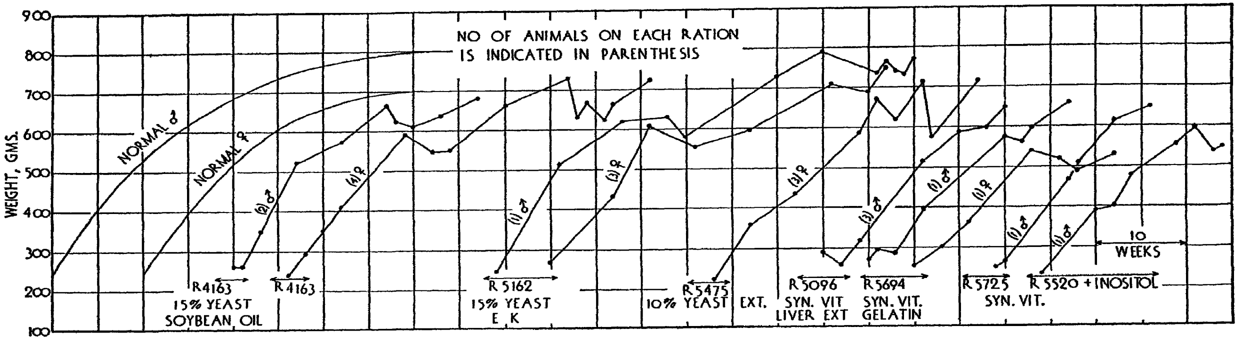

Fig. 1 A simplified ration that contains 15% of yeast, or 10% of yeast extract, supplied all water-soluble vitamins required by guinea pigs. All of the unrecognized vitamins of this group are supplied by a water extract of liver. Diets that are completely "synthetic," such as 5520, 5725, or 5694, are partially inadequate.

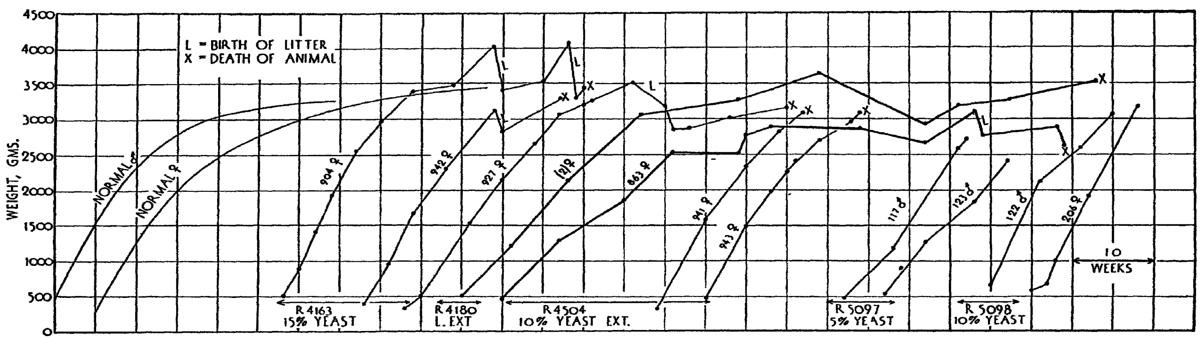

Fig. 2 A simplified ration that contains 10% of a yeast extract, or from 5 to 15% of yeast, supplied all water-soluble vitamins required by rabbits. Females were moderately successful in rearing their litters. All of the unrecognized vitamins required by these animals are supplied by liver extracts. The graph which shows the response to ration 4180 was drawn from the average weight of two animals.

normal in appearance and in rate of growth. Four females were grown to maturity on ration 4163 but it was partially inadequate for reproduction and was changed to a ration identical with no. 5162 except that it contained 0.2% choline. After this change was made these four females bore six litters containing fifteen young that were alive at birth and two that were dead. Thirteen of the young survived until weaning age. The three females continuously on ration 5162 bore six litters containing a total of seventeen young, and of these thirteen were weaned. The rate of growth on ration 5475, which contained 10% of the yeast extract, was satisfactory but we were not certain that this amount was adequate during reproduction. Two litters of three young each were weaned, but another litter of two was aborted.

As will be mentioned later, an attempt was made to rear guinea pigs on diets that contained the vitamins only in the pure form. The effort was not a complete success, however, and in a search for useful carriers, liver extracts were included in some of these diets, as in ration 5096. The three males which received this diet did not consume it readily for the first 2 weeks, but after that time they grew exceptionally well.

The response of the rabbits is shown in figure 2. The animals grew at the normal rate and no nutritional deficiencies were observed. Three females which received ration 4163 bore a total of four litters, with seventeen living young. Since it is our practice to reduce each litter to six, fifteen had an opportunity to survive. Of these young seven were weaned, giving a mortality of 53%.

Of the three females on ration 4504, which contains a water extract of yeast, two did not survive sufficiently long to bear litters. The other, rabbit 863, bore eight young in two litters and weaned five. She survived for approximately 18 months.

Rations 5097 and 5098 were prepared to show how much yeast the rabbit requires. The two males on ration 5098, which contains 10% of yeast, grew at the normal rate. One of the animals on ration 5097 grew almost as rapidly, but the growth

rate of the other was appreciably retarded. It is concluded that the optimum amount for growth lies between 5 and 10%.

Ration 4180 contains 10% of liver extract and the average weights of the two animals which received it are shown in figure 2. The growth rate equals that of the animals in the stock colony, and these two females bore a total of six litters containing forty living and seven dead young. Of the forty born alive thirty-one were retained and fifteen were weaned, a mortality of 52%. The mortality in the stock colony has been about 33%.

When it was discovered that rats can be grown on simplified rations that contain vitamins in pure form, with no crude vitamin carriers (Richardson, Hogan, Long and Itschner, '41) it was decided to try a similar diet on guinea pigs. Though encouraging, these attempts have not been very successful. Typical rations of this type are described in table 2, and a few of our data are summarized in table 3 and in figure 1.

TABLE 2

Experimental diets that contain no crude vitamin carriers.

RATION NO.	5520	5725	5694
	gm.	*gm.*	*gm.*
Casein	20	20	20
Gelatin			10
Dextrin	39	39	39
Sucrose	10	10	
Cellulose	15	15	15
Lard	10	10	10
Salts	4	4	4
A–D mixture	2	2	2
	mg.	*mg.*	*mg.*
Thiamine	0.8	0.8	0.8
Riboflavin	1.6	1.6	1.6
Pyridoxine	1.2	1.2	1.2
Ca-pantothenate	2.0	2.0	2.0
Nicotinic acid	2.0		2.0
Choline	300.0	300.0	300.0
Alpha-tocopherol	4.0	4.0	2.0
2-methyl-1, 4-naphthoquinone	2.0	2.0	2.0
p-amino benzoic acid	30.0		20.0
Inositol			50.0

Table 3 indicates that ration 5694 is more adequate than any other of this type yet tried. If it is superior to the others we are inclined to ascribe the difference to inositol, though the inclusion of gelatin may be of some significance. Of the eight animals on this diet, the growth of three is reasonably good and that of two is definitely unsatisfactory; three exhibited

TABLE 3

Growth of guinea pigs on diets that contain no crude vitamin carriers.

RATION NO.	ANIMAL	PERIOD OBSERVED	WEIGHT		NOTES
			Initial	Final	
		weeks	*gm*	*gm.*	
	149F	15	260	395	unthrifty, changed
5520	216M [1]	16	251	595	thrifty
	224F [1]	3	264	305	thrifty
	149F	9	390	545	thrifty
	209M	18	271	630	thrifty
	210F	18	250	540	thrifty
	236F	17	249	450	thrifty
5694	238M	17	239	585	thrifty
	202F	6	210	290	died, cause unknown
	237F	4	259	315	died, cause unknown
	239F	6	211	280	unthrifty, discontinued
	219M	16	256	655	thrifty
	240F	10	246	350	unthrifty, discontinued
5725	261F	5	266	375	thrifty
	222F	8	245	285	died
	223M	8	250	345	unthrifty, discontinued

[1] Received 10 mg. daily of inositol, supplied separately.

failure of growth. The superior performance of the two animals on ration 5520 supplemented with inositol lends support to the view that this compound may be significant.

Since rats grow normally on diets similar to those shown in table 2, while guinea pigs fail, it would seem that aside from vitamin C the nutritional requirements of the guinea pig are more complex than those of the rat. Presumably this complexity is in some way related to the synthesis, and utilization, of vitamins that are synthesized by bacteria of the intestinal tract.

Whatever may be the deficient nutrient in the rations described in table 2 it is abundantly present in extracts of liver. Ration 5096 for example contains 4% of our extract 4303, soluble in alcohol, and 6% of the water soluble portion, extract 4080. The animals grow at a normal rate on this ration. Rabbits were used in our first studies with the liver extracts, but as their rations were somewhat more complicated than those mentioned they are not described in detail. Six rabbits were reared on these rations though, with no difficulty. Two of these together bore five litters, containing thirty-seven young alive at birth. Twenty-nine were retained, and seventeen were weaned. This record compares favorably with that of those on the stock diet.

SUMMARY

1. Guinea pigs and rabbits grow at the normal rate on simplified rations which contain dried yeast as the source of all water-soluble vitamins. A water extract of dried liver also contains the essential water-soluble vitamins that are not yet recognized.

2. When vitamin K was added to the simplified diets the females were able to rear litters.

3. When all water-soluble vitamins were supplied as pure compounds now available, the mortality was high and the rate of growth was subnormal.

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RELATIONSHIP BETWEEN PANTOTHENIC ACID REQUIREMENT AND AGE IN THE RAT

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TWO FIGURES

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In previous studies on the requirement for pantothenic acid in albino and piebald rats (Unna, '40; Unna, Richards and Sampson, '41) the daily maintenance dose for optimal growth was found to be between 80 and 100 μg. per rat per day. This figure, since confirmed by Henderson, McIntire, Waisman and Elvehjem ('42) and C. A. Slanetz [1], was established by experiments on rats of 3 weeks of age. In weanling rats subsisting on a sub-optimal amount of pantothenic acid (40 μg. daily), the appearance of deficiency lesions was observed during the first 6 weeks of the dietary regimen, but spontaneous regression of the lesions began after the sixth week. Similar observations were made on young black and piebald rats; daily amounts of 25 or 50 μg. of calcium pantothenate, respectively, were insufficient to prevent greying of the fur, but after a period of 7 weeks, without any change in the daily allowance of pantothenic acid, gradual restoration of the black color of the fur became noticeable. Similar observations have been reported in a recent paper by Henderson, McIntire, Waisman and Elvehjem ('42). Thus, with doses representing about one-half of the requirement, regression of the deficiency lesions was observed when the rats reached the age of 9 to 10 weeks. At this time, their weight had increased from 30 gm.

[1] Personal communication.

at the beginning of the experiment to around 125 to 150 gm., and their food consumption had more than doubled. These observations suggested the possibility that the rat's requirement for pantothenic acid decreases with progressing age, and the question arose whether a relation could be established between the demand for pantothenic acid and the stage of life of the rat. Experiments intended to shed light on this problem are reported in this communication.

All animals were maintained on a vitamin B complex free diet having a percentage composition of dextrose, 68; casein, vitamin free, 18; hydrogenated cottonseed oil [2], 8; salt mixture USP XI No. 1, 4; cod liver oil, 2; supplemented with 0.8 mg. each of thiamine, riboflavin and pyridoxine, 10 mg. of nicotinamide and 100 mg. of choline chloride per 100 gm. of diet. Pantothenic acid in the form of aqueous solutions of crystalline dextrorotatory calcium pantothenate was administered daily in the desired dosage by stomach tube. The rats were kept in individual cages in an air-conditioned room (temperature 74° F. ± 2, humidity 45%). Weights were recorded at weekly intervals and the food consumption was measured daily whenever indicated.

Requirement at 3, 5 and 7 weeks of age

Ninety male albino rats placed on the basal diet at 3 weeks of age were divided into three series (A, B, C) of thirty rats each. These series were further subdivided into five groups of six animals each, and the same scheme of feeding graded doses of pantothenic acid was carried out for each series; a daily supplement of 0, 10, 25, 50 or 100 µg. of pantothenic acid, respectively, was administered to the animals of the five groups. In series A the feeding of graded doses was started at the beginning of the experiment. In the two corresponding series the animals were given 100 µg. of pantothenic acid for the first 14 or 28 days, respectively; feeding of graded doses was begun at 5 weeks of age (series B) or 7 weeks of age

[2] Crisco.

(series C). The average weight curves of the various groups are shown in figure 1.

A comparison of the corresponding groups in the three series of experiments shows that at 3 weeks of age withdrawal of pantothenic acid leads to the death of half of the group within 46 days; at 5 weeks of age, within 89 days; and at 7 weeks of age, within 146 days (fig. 1, curves 0). Deficiency lesions such as thinning of the fur, rusty spots and red tinged whiskers caused by deposition of porphyrin (McElroy,

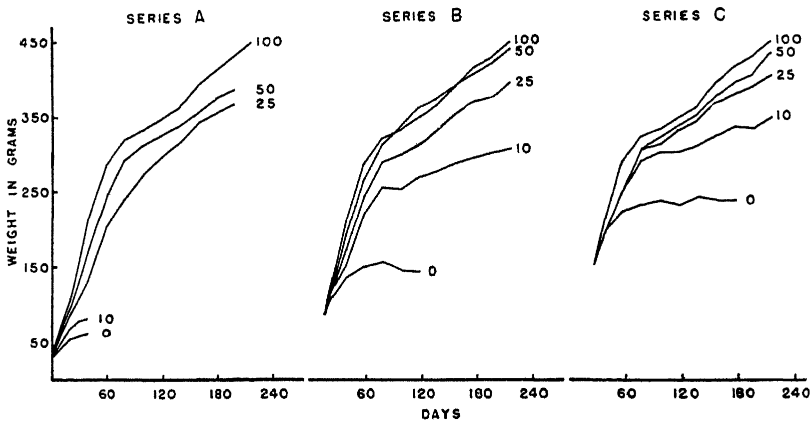

Fig. 1 Effect of daily feeding of pantothenic acid to rats of different ages maintained on a basal diet free from pantothenic acid. Series A: Daily feeding of graded doses (0, 10, 25, 50 and 100 μg.) of pantothenic acid, started at 3 weeks of age. Series B: Daily feeding of graded doses of pantothenic acid. Started at 5 weeks of age. Series C: Daily feeding of graded doses started at 7 weeks of age.

Salomon, Figge and Cowgill, '41) were observed in all groups deprived of pantothenic acid; they appeared after 2 weeks in the rats of series A and only after 4 weeks in the rats of series C. Adrenal hemorrhages were found on gross examination in rats of all groups.

Daily feeding of 10 μg. of pantothenic acid failed to support life of the majority of the rats started at 3 weeks of age. However, the same amount of pantothenic acid fed to rats of 5 and 7 weeks of age was sufficient to prevent death

and enabled these animals to gain weight continuously for the period of 220 days. Deficiency lesions, although present in all groups, were much more pronounced in the animals of series A than in those of the series B and C. In series A these lesions became manifest after 2 weeks, and adrenal hemorrhages were found on autopsy; in series C loss of hair together with some rusty discoloration of the fur became noticeable only after 10 weeks and no adrenal hemorrhages were observed on gross examination.

Daily feeding of 25 μg. of pantothenic acid was sufficient for the sustainment of life of all rats of the different age groups. The rats in series A exhibited after 5 weeks a coarse and scant fur, but this manifestation regressed after 125 days. Rats in series B showed comparable signs of deficiency, disappearing gradually after 7 weeks. The rats in series C, however, remained entirely free from these lesions throughout the experiment, and, furthermore, their weight curve approximated those of the rats receiving daily amounts of 50 or 100 μg. of pantothenic acid. The rats in series A receiving a daily supplement of 50 μg. of pantothenic acid developed a somewhat thin and coarse coat which lacked the luster and smoothness of normal fur. However, this manifestation was transient and at the end of the experiment their coats matched those of the group receiving 100 μg. of pantothenic acid. No impairment of the coat was observed in the corresponding groups in the series B or C. A comparison of the growth curves of the groups receiving 50 and 100 μg. of pantothenic acid demonstrates that 50 μg. is inadequate to ensure optimum growth in rats started at 3 weeks of age. However, in the corresponding groups of 5 and 7 weeks of age, no significant difference could be found in the weight curves, a daily dose of 50 μg. of pantothenic acid being adequate for optimal growth.

Requirement at 10 weeks of age

Male and female albino as well as piebald rats (total number 170) of approximately 10 weeks of age were used for these experiments. The animals weighing between 150 and

225 gm. were placed on the basal diet described on page 546; prior to the experiments, the animals were maintained on the usual laboratory stock diet. The amounts of pantothenic acid fed daily to these rats were 0, 10, 25 and 100 µg. respectively. Figure 2 demonstrates the changes in weight observed in a representative group of these experiments.

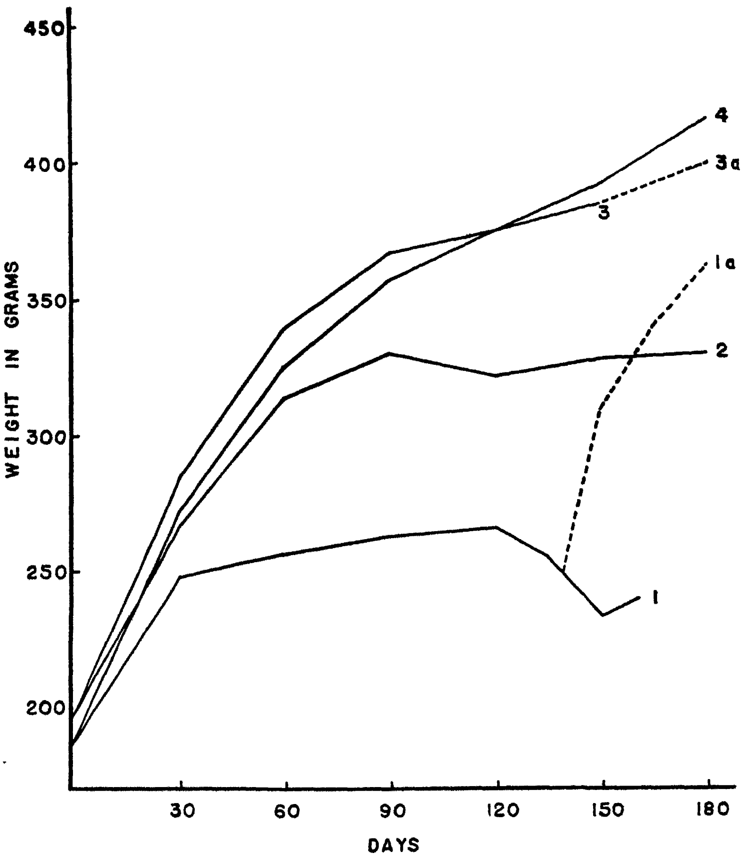

Fig. 2 Effect of daily feeding of graded doses of pantothenic acid to rats of approximately 10 weeks of age maintained on a basal diet free from pantothenic acid. (1) No pantothenic acid; (2) 10 µg. of pantothenic acid daily; (3) 25 µg. of pantothenic acid daily; (4) 100 µg. of pantothenic acid daily; (1 a) Daily feeding of 200 µg. of pantothenic acid; (3 a) Daily feeding of 100 µg. of pantothenic acid.

Following the withdrawal of pantothenic acid at 10 weeks of age, the rats continued to gain weight during a period of 1 month at about the same rate as those receiving ample amounts of the vitamin. Thereafter, their weight became stationary (fig. 2) and deficiency lesions became manifest. The lesions progressed in severity throughout the experiments. Some animals succumbed after 3 months of deprivation of pantothenic acid, but two-thirds of all rats (forty-three) in this series survived for more than 5 months. On autopsy gross adrenal hemorrhages were found in only three animals; the incidence of adrenal hemorrhages, therefore, was much less than in the young rats of 3 weeks of age in which adrenal hemorrhages were observed in about 50% of all autopsies. In black rats greying of the fur was noticeable during the second month; most of the animals became completely grey after a period of 4 months.

Daily feeding of 10 µg. of pantothenic acid supported the life of all rats for more than 6 months. The animals continued to gain weight during the first 2 months (fig. 2). At this time, their coats became roughened and coarse with some rusty discoloration, and their weights remained stationary. In a group of eight black rats, only one animal became markedly grey.

Daily feeding of 25 µg. of pantothenic acid produced the same results as daily supplements of 100 µg. No signs suggestive of pantothenic acid deficiency were found in either albino or black rats of these groups. The weight gains of the animals receiving 25 µg. were identical with those receiving 100 µg. of pantothenic acid (fig. 2), demonstrating that 25 µg. of pantothenic acid per day is the approximate maintenance dose for optimal growth in rats 10 weeks of age. After a 6-month period, male albino rats reached average weights of 400 to 450 gm., females 230 to 270 gm.

Correction of deficiency lesions in adult rats

Rats placed on the pantothenic acid free diet at 10 weeks of age responded, after deprivation periods of 3 and 5 months

or more, to treatment with pantothenic acid in doses of 100 or 200 μg. per day with striking weight gains and improvement of the deficiency lesions. Within 4 to 6 weeks their general appearance equaled that of the animals maintained on adequate amounts of pantothenic acid. In black or piebald rats restoration of the black color of the fur was obtained within 2 months, a period about twice as long as that required in rats of three weeks of age (Unna, Richards and Sampson, '41). When treatment was initiated after a deprivation period of 3 months, the animals attained, within 30 days, the average weight of those existing on adequate amounts of pantothenic acid; after a deprivation period of 5 or 6 months about 2 months of treatment were required to regain the normal weight (fig. 2, curve 1 a).

In other experiments, rats started at 10 weeks of age and subsisting for 3 months on 10 μg. of pantothenic acid daily were dosed with 25 μg. daily. Within 1 month, all signs of the deficiency disappeared and their weights recovered the levels attained by the control rats fed adequate amounts of pantothenic acid throughout the entire period. On the other hand, an increase of a daily supplement of 25 μg. to 100 μg. failed to raise the weight curve (fig. 2, curve 3 a); additional confirmation that a daily supplement of 25 μg. meets the requirement for optimum growth in adult rats.

DISCUSSION

The data presented here demonstrate that the daily maintenance dose of pantothenic acid for optimum growth in rats decreases greatly with progressing age of the animal. In weanling rats consuming 5.3 gm. of diet daily it is approximately 80 to 100 μg., whereas in adult rats with an average food consumption of 15.5 gm. daily, it amounts to only 25 μg. Observations on the appearance of deficiency lesions corroborate the findings on growth; the daily amount of pantothenic acid necessary to prevent these lesions is 100 μg. in very young rats and only 25 μg. in rats of 10 weeks of age. These findings were the same with two different strains, albino and

piebald rats and, furthermore, were obtained alike in male and female animals.

Storage of pantothenic acid might be considered as a possible factor involved in the difference of the response between younger and older rats. Since it has been demonstrated previously that at the age of 3 weeks a daily supplement of 100 μg. of pantothenic acid just meets the requirement of the rat for maximum growth, a possibility for a storage of the vitamin at this age appears to be slight and; certainly would not offer a satisfactory explanation for our findings. Hence, we feel that the striking change in the requirement for pantothenic acid is related to the age of the animals. The sharp decline in the requirement takes place between the third and the tenth week of the life of the rats. It coincides with the period in which the rapid growth rate of the infant rat is gradually declining. Also, the experiments demonstrate clearly that, at least during these stages of life, the pantothenic acid requirement bears no relation to the weight nor to the food consumption of the animal. In this respect our findings with pantothenic acid are a diametrical counterpart to the findings with thiamine by Osborne, Mendel and Cannon ('22), Cowgill ('34) and Jolliffe ('38). This striking decrease in the requirement during a period of great increase in food consumption and in weight is quite the reverse of the approximately linear relationship of the thiamine requirement to body weight (Cowgill, '38) or to non-fat calories (Williams and Spies, '38).

Additional evidence for a difference between the requirement of pantothenic acid and that of thiamine is found in the different response of depleted rats to single curative doses. The thiamine deficient rat reacts to a single dose of thiamine with a sharp but transient weight response; its weight returns to its original level almost as rapidly as it had increased (Smith, '30) in response to the vitamin administration. The pantothenic acid deficient rat responds to a single curative dose of pantothenic acid with a similar increase in weight, but the weight gain is sustained, and subsequent doses at

weekly intervals produce a "staircase" effect in the weight curve, as previously described (Unna, '40).

The observation that the greatest need for pantothenic acid coincides with the period of maximal growth indicates that pantothenic acid may play a role in metabolism quite different from that of thiamine and other B vitamins connected with carbohydrate metabolism, and permits the speculation that it may be connected with metabolic processes involved in the formation of new tissue.

SUMMARY

The pantothenic acid requirement at various stages of life was studied in albino and piebald rats maintained on a basal diet devoid of pantothenic acid.

1. The maintenance dose for optimal growth and for prevention of characteristic deficiency lesions decreases from 100 µg. per day at 3 weeks of age to 25 µg. per day at 10 weeks of age.

2. The requirement for pantothenic acid cannot be correlated with the weight or the food consumption of the rat.

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THIAMINE, RIBOFLAVIN, PYRIDOXINE AND PANTOTHENATE DEFICIENCIES AS AFFECTING THE APPETITE AND GROWTH OF THE ALBINO RAT[1]

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ONE FIGURE

(Received for publication January 12, 1942)

This report deals with an investigation of moderate vitamin deficiencies such as are likely to occur in human dietaries. The results presented herein, relating to appetite and growth, will be followed by associated observations on the composition of the body gains.

Growth effects of thiamine, riboflavin, pyridoxine and calcium pantothenate were determined separately by use of the paired-feeding method wherewith a vitamin-fortified basal diet was compared simultaneously with similar diets differing only with respect to one of the vitamins.

Appetite effects were determined by comparison of the food consumption of the rats on the deficient diets with the food consumption of rats receiving the complete diet ad libitum.

The beneficial effect of thiamine on growth of rats has been found to be largely the result of its effect on appetite (Hogan and Pilcher, '33; McClure, Voris and Forbes, '34; Voris, '37; Harris, '38; Schrader and Prickett, '38).

A claim that thiamine has a specific effect on growth unrelated to food intake was advanced by Sure and his collabo-

[1] Authorized for publication on January 9, 1942, as paper no. 1076 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

rators (Sure, Kik and Smith, '32; Sure, '32; Sure, Kik, Walker and Smith, '33), but this conclusion was recently modified by Sure and Dichek ('41 a) who reported that rats receiving suboptimal levels of thiamine grew as well as pair-fed rats receiving ample thiamine; however, when the deficiency of thiamine was extreme, the deficient rats lost weight more rapidly than their pair-mates receiving ample thiamine.

This latter observation was not confirmed by Voris and Thacker (unpublished results) who found that rats receiving thiamine lost weight at the same rate and failed to survive as long as their deficient pair-mates. It was concluded that the lessened vital activities of the deficient rats, associated with a reduced basal metabolism (Voris, '37), bradycardia, etc., accounted for their ability to withstand the submaintenance conditions for a longer period than their controls.

Riboflavin deficiency has been found to affect growth specifically but not the appetite of rats (Sure and Dichek, '41 b; Sure, '41). According to these investigators, the anorexia accompanying extreme riboflavin deficiency was relieved by the administration of pyridoxine.

The present authors are unaware of any studies which have dealt with the specific effects of either pyridoxine or calcium pantothenate on growth with controlled feed consumption.

EXPERIMENTAL

The basal diet used for these studies had the following percentage composition: sucrose 68, casein [2] 20, hydrogenated vegetable oil [3] 5, a cellulose preparation [4] 2, salt mixture [5] 3, cod liver oil [6] 2.

Supplementary factors were added to this diet to make up the test rations. The designation of the rations and the

[2] Labco, vitamin-free.

[3] Crisco.

[4] Cellu flour from Chicago Dietetic Supply House, Chicago, Illinois.

[5] Hubbell, Mendel and Wakeman, '37.

[6] E. R. Squibb and Son, medicinal grade.

quantities of supplements provided per gram of ration were as follows:

Ration A (complete) — thiamine 10 μg., riboflavin 10 μg., pyridoxine 5 μg., Ca pantothenate 20 μg., choline 4 mg., B alanine 10 μg., nicotinic acid 10 μg., α tocopherol 25 μg., and inositol 1 mg.

Ration B (low thiamine) — thiamine 1 μg., and all other supplements the same as ration A.

Ration C (low riboflavin) — riboflavin 1 μg., and all other supplements the same as ration A.

Ration D (low pyridoxine) — pyridoxine 0.5 μg. and all other supplements the same as ration A.

Ration E (low pantothenate) — Ca pantothenate 2 μg. and all other supplements the same as ration A.

Ration A_1 (liver extract) — Ration A plus 0.2% liver extract.[7]

Ninety-five weanling albino rats of the Wistar strain were used as experimental subjects. These were distributed as follows: ten litter mate pairs (five male, five female) for the comparison of ration A with each one of rations B, C, D and E; five litter mate pairs (female) for comparison of ration A_1 with ration A and for testing the adequacy of ration A for growth when fed ad libitum; five rats (female) for ad libitum feeding of ration A_1 in order to ascertain its adequacy for growth in comparison with the rats fed ration A ad libitum.

With the exceptions indicated, the paired-feeding technic was employed, and the quantity of feed allotted was determined daily by the rat of a pair which consumed the lesser quantity of feed. The feed was weighed on a precision balance to the nearest 0.1 gm. The rats were kept in individual cages with feeders and watering-bottles as described by Swift, Kahlenberg, Voris and Forbes ('34). Tap water was allowed ad libitum. All rats were weighed once a week throughout the 10 weeks experimental period.

In the fourth and fifth weeks rats on the thiamine test were given 5 μg. daily of additional thiamine, and throughout the last 5 weeks they were given 3 μg. of thiamine daily in addition to the thiamine in the rations. The thiamine was mixed with a cellulose preparation[8] so that 1 gm. of the

[7] Eli Lilly & Co.

[8] See footnote 4, page 556.

mixture contained 50 µg. of the vitamin. Weighed portions of the mixture providing the allotment of supplementary thiamine were added to the daily feed for both rats of a pair. This thiamine supplementation became necessary after the third week because the rats receiving ration B (low thiamine) were limiting the feed consumption to submaintenance levels.

Likewise, it became necessary to provide additional Ca-pantothenate to four rats receiving ration E. One pair was given 10 µg. daily of additional pantothenate in a cellulose preparation [9] after the third week and three other pairs were given the same after the fourth week.

No supplementation was necessary for the rats on rations C or D.

After 10 weeks on the experimental rations the rats were killed with illuminating gas, the contents of the alimentary tracts removed and the empty weights obtained. The empty weights at the start of the experiment were estimated from the average alimentary fill of corresponding rats killed for this purpose.

The temperature of the room in which the rats were kept averaged 25°C. during the first 6 weeks and 23°C. during the last 4 weeks. These temperatures were fairly constant for the periods designated, rarely deviating as much as a degree from the averages given. The lower temperature during the last 4 weeks resulted when the use of a heating unit in the room was discontinued.

RESULTS

Rations A and A_1 (complete, and same plus liver extract). These rations were fed ad libitum to female rats only and the average gain for 10 weeks was 167.4 gm. for the rats receiving ration A and 168.1 gm. for those receiving ration A_1 (table 1). Maximum weekly gains of 29.6 gm. and 30.0 gm. were made during the second and third weeks without much decrease in

[9] See footnote 4, page 556.

the rate of gain until after the sixth week. The rate of gain of the rats on these synthetic rations compared favorably with that of the stock colony rats from which they were derived. The addition of liver extract did not improve the adequacy for growth of the completely synthetic ration A.

TABLE 1

The influence of suboptimal intakes of thiamine, riboflavin, pyridoxine and pantothenate on growth of rats pair-fed with rats on a completely fortified synthetic ration for 10 weeks.

RATION	NO. OF RATS AND SEX	FEED INTAKE	VITAMIN INTAKE	FINAL WEIGHT[1]	INITIAL WEIGHT[1]	GAIN	FEED FOR 1 GM. GAIN
		gm.	*µg./day*	*gm.*	*gm.*	*gm.*	*gm.*
			Thiamine				
B: low thiamine	5 ♂	549	10	183	38	145	3.8
A: complete	5 ♂	550	81	182	37	144	3.8
B: low thiamine	5 ♀	545	10	149	39	110	5.0
A: complete	5 ♀	545	80	161	40	121	4.5
			Riboflavin				
C: low riboflavin	5 ♂	718	10.3	217	43	174	4.1
A: complete	5 ♂	718	103	240	43	197	3.7
C: low riboflavin	5 ♀	598	8.5	167	39	128	4.7
A: complete	5 ♀	598	85	175	40	135	4.4
			Pyridoxine				
D: low pyridoxine	5 ♂	587	4.2	189	43	146	4.0
A: complete	5 ♂	589	42	202	42	160	3.7
D: low pyridoxine	5 ♀	498	3.6	131	38	93	5.4
A: complete	5 ♀	498	36	135	38	97	5.1
			Pantothenate				
E: low pantothenate	4 ♂	597	19	183	43	140	4.3
A: complete	4 ♂	597	172	202	44	159	3.8
E: low pantothenate	5 ♀	547	18	148	38	110	5.0
A: complete	5 ♀	547	159	164	38	126	4.3
A: complete	2 ♀	755		213	47	166	4.5
A_1: A + liver extract	2 ♀	755		209	47	162	4.7
A: *ad libitum*	5 ♀	749		213	45	167	4.5
A_1: *ad libitum*	5 ♀	759		209	41	168	4.5

[1] Excluding contents of alimentary tract.

With respect to the response of male rats to ration A it may be noted that those receiving this ration in the paired groups gained 1 gm. in weight for each 3.72 gm. of feed consumed, whereas the female rats required 4.57 gm. of feed for each gram gained. The male rats receiving ration A paired with those receiving ration C (low riboflavin) gained 196.8 gm. on 718 gm. of feed as compared with a gain of 167.4 gm. on 749 gm. of feed made by the female rats receiving ration A ad libitum.

In planning this investigation, the idea was considered that the rats receiving ration A ad libitum would limit the feed consumption of paired rats receiving ration A_1. However, only two rats on ration A_1 would eat as much as their pair-mates on ration A and, consequently, there were only two paired comparisons of these rations. The rats on ration A gained 166.3 gm. as compared with 162.4 gm. gained by their pair-mates on ration A_1 during the 10 weeks.

Ration B (low thiamine). With 1 μg. of thiamine per gram of feed, the rats receiving ration B exhibited marked anorexia during the third week on the ration (fig. 1) and the feed consumption dropped to about 4 gm. daily, on the average, with consequent loss of weight. With an additional 5 μg. of thiamine daily during the fourth and fifth weeks the feed consumption of the rats increased to 9 gm. daily and the gains in weight averaged 3.5 gm. daily.

Since this allowance of thiamine appeared to be excessive for the purposes of the experiment, no additional thiamine was given in the early part of the sixth week but after the second day the feed consumption had dropped to 5 gm. daily so that it was necessary to provide an additional 3 μg. of thiamine daily. This supplement in addition to the thiamine in the ration permitted positive growth through the ninth week. The feed intake during the ninth week had decreased about 10% and during the tenth week it dropped about 40% compared with the feed consumption of the seventh and eighth weeks.

Thus, it is obvious that 1 μg. of thiamine per gram of feed was just insufficient to maintain the appetites of the rats. They responded with remarkable sensitivity to slight adjustments of the thiamine intake by supplementation, but without supplementation the feed consumption was insufficient for maintenance.

The growth curves (including both sexes) of the paired rats used for comparison of rations A and B were practically

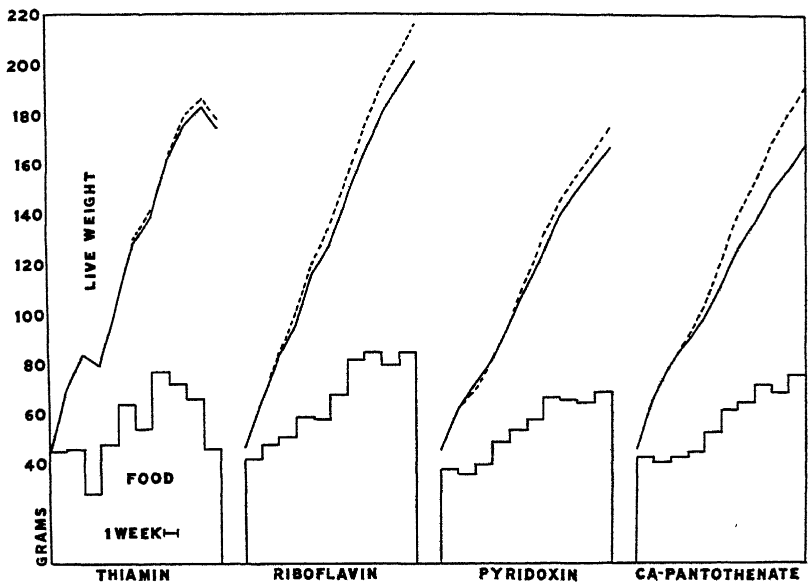

Fig. 1 Growth and food consumption of pair-fed rats comparing the complete synthetic ration A (dotted lines) with rations B, C, D and E (solid lines), each deficient in only one of the vitamins. Curves include equal numbers of males and females.

parallel (fig. 1). The slight weight advantage accruing to the rats receiving ration A was confined entirely to the females. The male rats on rations A and B gained an average of 144.2 and 144.7 gm., respectively. The female rats receiving ration A gained 120.9 gm. and their pair-mates receiving ration B gained 109.8 gm. as an average. There were no exceptions among the female rats in this respect and statistically the

odds are 2000 to 1 against this difference occurring by chance alone. For the group as a whole, the odds are only 18 to 1 that thiamine had a significant effect on growth.

Ration C (low riboflavin). With 1 µg. of riboflavin per gram of feed, the rats receiving ration C limited the feed intakes of their pair-mates receiving ration A without exception during the first 7 weeks and 60 against 9 times during the last 3 weeks.

The appetite depression with insufficient riboflavin was dissimilar in magnitude and character to that occurring with thiamine insufficiency. In the latter case the appetite was likely to drop to disastrous levels and was quite sensitive to slight changes in thiamine intake. With riboflavin insufficiency the appetite depression was mild in degree and relatively consistent, appearing as characteristic of a general rather than a specific effect. Also, there was a marked difference in feed consumption between the male and female rats receiving ration C, an effect which did not appear in the low thiamine group.

After the second week the weight gained by the rats receiving ration C was consistently less than that gained by their pair-mates receiving ration A (fig. 1).

In 10 weeks the male rats receiving ration C gained 88.2% and the female rats 94.5% as much weight as their pair-mate controls on ration A. The growth difference favoring ration A was without exception among the pairs of rats and was definitely significant in indicating a specific effect of riboflavin in the economy of feed utilization separate from its effect on appetite. The odds were 3000 to 1 against the difference occurring by chance.

Ration D (low pyridoxine). A depression of appetite with insufficient pyridoxine was evident within the first week of feeding ration D, and throughout the 10 weeks the rats receiving this ration limited the feed intake of their pair-mates receiving ration A without a single exception. The character of the appetite depression was different from that observed with an insufficiency of either thiamine or riboflavin. On low

pyridoxine intake the appetite of a rat would have a sudden and temporary lapse with refusal of a large portion of the daily feed followed by a return to the former level of feed consumption within a day or so.

A slight retardation of growth of the low pyridoxine rats was evident after the third week (fig. 1). The difference was more marked with the male than with the female rats. The male rats receiving ration D gained 90.8% and the female rats 95.6% as much weight as their control pair-mates receiving ration A. The growth advantage favoring ration A was evident in all pairs of the male rats but among the female pairs one control rat gained less weight than the low-pyridoxine mate, and in one pair the gains were equal. The odds were 50 to 1 against the difference occurring by chance alone.

Ration E (low pantothenate). The appetite depression evident with the rats receiving ration E persisted throughout the 10 weeks. Three of the ten pairs of rats had to be given additional pantothenate to prevent loss of weight because of insufficient feed consumption. Two rats (one male, one female) receiving ration E developed typical pantothenate deficiency symptoms (reddening of hair about head, caked whiskers and sticky, unkempt fur). These rats were from the same litter. The symptoms disappeared in the female with the additional 10 μg. of Ca pantothenate daily but persisted throughout the last 7 weeks in the male rat. The latter rat developed the habit of scattering feed during the last 2 weeks and the pair of which he was a member was not included in the average results of table 1.

After the third week the rats receiving ration E gained consistently less than their pair-mates receiving ration A (fig. 1). This group showed a larger divergence in weight than any of the other groups. The difference in the response of the sexes was not as marked as with the low riboflavin and low pyridoxine groups. The male rats receiving ration E gained 87.8% and the female rats 87.3% as much weight as

their pair-mates on ration A. The odds were 200 to 1 against the difference occurring by chance alone.

DISCUSSION

Mills ('41) reported positive growth with rats on a diet containing as little as 0.6 μg. of thiamine per gram in an environmental temperature of 32°C. and as little as 0.4 μg. per gram in a temperature of 18°C. It has been noted in the present investigation that 1 μg. of thiamine per gram of ration was insufficient for maintenance. The diet used by Mills contained a larger proportion of carbohydrate than ration B but also contained 2% of a liver extract which possibly contained some thiamine. The slight difference in average temperature for the first 6 weeks (25°C.) as compared with the last 4 weeks (23°C.) in the present investigation had no apparent effect on the thiamine requirement of the rats.

It is obvious that all the factors investigated herein have a definite effect on appetite. A critical quantitative comparison of the different factors with respect to their effect on appetite is not feasible from the present data since graded responses to different levels of intake could not be included. However, for the particular differences in vitamin intake as imposed in these tests, the feed consumption of the female rats on the low levels of intake compared with the female rats fed ration A ad libitum was reduced 27% for ration B (low thiamine), 20% for ration C (low riboflavin), 34% for ration D (low pyridoxine), and 27% for ration E (low pantothenate).

The qualitative differences in appetite effects based on daily observation and impressions supported by the daily feeding records may be noteworthy. With insufficient thiamine the appetite of the rat would drop to submaintenance levels and the disinterest in eating would continue until additional thiamine was provided. With insufficient riboflavin the appetite depression appeared to be the result of a general physiological debility of an unspecific nature. With insufficient pyridoxine there were sudden and temporary lapses in appetite followed by an equally prompt recovery. Pantothenate

anorexia seemed to follow the pattern of riboflavin insufficiency.

The results presented herein indicate that riboflavin, pyridoxine and pantothenate have specific growth-promoting effects unrelated to appetite. Thiamine does not seem to have a specific effect on growth separate from its effect on appetite when the sexes are not differentiated. However there was a favorable effect of thiamine indicated in body weight gained by female rats.

SUMMARY

The effects on the appetite and growth of rats with moderate deficiencies of thiamine, riboflavin, pyridoxine and calcium pantothenate were investigated by comparing a complete synthetic diet with similar diets differing only with respect to one of the vitamins. The paired-feeding method was used to distinguish the appetite and growth effects.

All four vitamins investigated had a definite effect on appetite, the character of the effect being somewhat different for each vitamin.

Riboflavin, pyridoxine and pantothenate had specific growth-promoting effects unrelated to appetite. Thiamine had no definite effect on the growth of male rats but improved the growth of female rats significantly.

Neither appetite nor growth was improved by the addition of liver extract to the so-called complete synthetic diet.

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THE EFFECT OF A HIGH VITAMIN A INTAKE ON THE BLOOD AND MILK CAROTENE OF HOLSTEIN AND GUERNSEY COWS [1]

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ONE FIGURE

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In an earlier paper from this laboratory (Deuel, Halliday, Hallman, Johnston and Miller, '41), it was shown that the vitamin A content of butterfat of Guernsey cows could be considerably increased if vitamin A in the form of shark liver oil was administered in daily amounts of 700,000 I. U. Smaller doses proved ineffective while larger amounts produced a greater augmentation in the vitamin A content of the butterfat. These responses occurred without the deleterious effect on milk production noted earlier when cod-liver oil was used as a supplement (Golding et al., '26).

A marked decrease in the level of carotene secreted in the milk occurred when the vitamin A intake was elevated by the feeding of shark liver oil, even when the quantity of the vitamin A fed was too small to cause a rise in the A content of the milk. Gallup ('41) has also recently reported that the daily administration of cod-liver oil to calves on winter rations caused about a 50% decrease in plasma carotene.

[1] This work was assisted by a research grant from the California Packing Corporation. Dr. J. P. Nutall and Dr. C. M. Bonynge of the Los Angeles Certified Milk Commission assisted in the planning of the experiments. The tests on the cows were made with the cooperation of the Adohr Milk Farms, Los Angeles.

These data were presented before the Symposium on Biochemistry in Section C, A. A. A. S. meeting in Dallas, December 29, 1941.

The present investigation was carried out to determine whether the depression in carotene is to be ascribed to either some toxic component in the shark-liver oil or the vitamin A per se. Also the effect of shark liver oil administration was studied in Holstein cows where the carotene metabolism normally proceeds at a level different from that in the Guernsey.

EXPERIMENTAL

The first series of experiments were carried out on twenty-five Guernsey cows of a large certified dairy, while twenty-six Holstein cows were employed in the second series of tests. The animals received a diet of approximately 5 pounds of a grain mixture which was fed twice daily together with alfalfa fed ad libitum. The control cows received only this basal diet while the experimental animals were fed a vitamin A supplement in addition. The tests were conducted simultaneously; therefore any differences in vitamin A or carotene secretion must be ascribed to the supplement.

The first series of tests were carried out from June 25 to October 6, 1941, on Guernsey cows. After a preliminary period of 2 weeks during which all the animals received only the basal diet, 30 cc. of shark liver oil [2] containing 700,000 I. U. of vitamin A (25,000 I. U. per gram) was administered over a 6 weeks' period to one group of eight animals in two equally divided doses daily, while a second group of eight cows received 700,000 I. U. of vitamin A concentrate assaying 545,000 I. U. per gram [3]. A third group of cows was maintained on the basal diet without supplement while one cow (no. 44) was fed shark liver oil at a much higher level. After this 6 weeks' period the supplement was discontinued and the animals were kept on the basal diet for a post-vitamin A supplement period

[2] Shark liver oil having a vitamin A potency of 25,000 U. S. P. units per gram was furnished by California Packing Corporation in the form of its Dairy Grade "Amin-A" Brand Oil. In the tests with Guernsey cow 44, 100,000 unit oil was employed.

[3] The vitamin A concentrate was a commercial sample obtained by molecular distillation.

until the carotene metabolism returned to normal. This required 7 weeks.

In the tests with Holstein cows a similar program was followed. This consisted of a basal period of 2 weeks for all animals followed by a vitamin A supplement period of 11 weeks during which shark liver oil in different amounts was given to two groups of eight cows; the remaining ten cows which served as controls received the basal diet without supplement. A post-vitamin A supplement period followed during which the diet was similar in the three groups and no supplement was administered. Ten weeks were required before the carotene returned to the normal level.

Milk samples were collected from each of two milkings on the first and fourth days in the weeks when analyses were made. The combined samples from each cow were mixed, soured overnight with a lactic acid bacilli culture, and churned on the following day. The butter was separated by pouring through cheesecloth; it was then washed, put in centrifuge tubes, melted, the water drawn off as thoroughly as possible and the fat filtered through anhydrous Na_2SO_4 into the sample bottles. As soon as the filtration was complete, the air above the sample was displaced with CO_2 and the samples were stored in a dark cold room until the analyses were carried out.

Vitamin A was determined on the non-saponifiable fraction by the use of the Bills-Wallenmeyer electronic photometer while later samples were also checked with the Beckman spectrophotometer. Both instruments were calibrated with the unsaponifiable fraction of reference cod-liver oil and corrections were made for absorption due to carotene.

Carotene was determined by the procedure of Koehn and Sherman ('40) with the Klett-Summerson photoelectric colorimeter, using a filter having a maximum transmission at 420 m.μ. Xanthophyll was removed by preliminary extraction. Blood carotene was determined on all animals at the period of greatest suppression of milk carotene as well as at the end of the test when the carotene metabolism had returned to

TABLE 1

The beta carotene and vitamin A per gram of butterfat in the milk of three groups of Guernsey cows during a basal period followed by a vitamin A supplement period when the basal diet was continued (control group) or when it was supplemented with 700,000 I. U. of vitamin A daily as 30 cc. of shark liver oil (group I) or as 1.4 cc. of a vitamin A concentrate (group II). No supplement was given to any group during post-vitamin A supplement period.

SAMPLE FROM WEEK STARTING	WEEK OF PERIOD	BETA CAROTENE IN MICROGRAMS			VITAMIN A IN INTERNATIONAL UNITS		
		Control Group	Group I	Group II	Control Group	Group I	Group II
				Basal Period			
June 25	1	9.48 ± 0.55	7.94 ± 0.51	10.20 ± 0.82	34.5 ± 3.6	31.4 ± 2.1	27.5 ± 2.3
July 2	2	9.78 ± 0.91	9.53 ± 0.50	10.09 ± 0.68	30.9 ± 2.8	26.5 ± 2.0	30.7 ± 3.4
Average		9.63	8.73	10.14	32.6	28.8	29.2
				Vitamin A Supplement Period			
July 16	2	8.79 ± 0.81	7.31 ± 0.69	9.33 ± 0.53	26.4 ± 1.6	44.3 ± 4.5	70.3 ± 3.0
						3.73	**12.92**
July 30	4	8.99 ± 0.94	5.81 ± 0.29	6.20 ± 0.53	28.2 ± 3.4 [1]	49.2 ± 3.5	67.8 ± 2.3
			3.25	**2.58** [2]	4.28	**4.28**	**9.65**
Aug. 13	6	9.68 ± 1.00	5.85 ± 0.37	5.43 ± 0.30	31.6 ± 2.7	70.3 ± 5.4	93.8 ± 1.9
			3.58	**4.08**		**6.44**	**18.85**
				Post-Vitamin A Supplement Period			
Aug. 25	2	8.16 ± 0.76	5.64 ± 0.27	5.50 ± 0.60	36.6 ± 3.8	46.6 ± 1.8	45.6 ± 3.3
			3.15	**2.77** [2]		**2.38** [2]	**1.80**
Sept. 8	4	8.17 ± 0.71	6.27 ± 0.39	6.60 ± 0.31	43.0 ± 2.5	36.9 ± 1.5	27.7 ± 1.8
Sept. 15	5	9.41 ± 0.92	7.99 ± 0.47	9.46 ± 0.58	33.9 ± 1.7	37.4 ± 2.9	38.0 ± 3.7
Sept. 29	7	9.04 ± 0.76	8.14 ± 0.49	8.64 ± 0.20	26.4 ± 0.8	30.2 ± 1.6	40.3 ± 2.9

The figures for beta carotene and vitamin A include values for the standard error of the mean calculated as follows: $\sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

The figures in bold face type are the quotients of the mean difference and the standard error of the mean difference. When this exceeds 3.00, the differences are considered statistically significant.

Averages of eight experiments in all cases except where noted.

[1] Seven experiments only.

[2] Based on another method of statistical evaluation (method II), these values are statistically different from those for the controls. When not more than 10% of the individual experiments of one group exceed or are less than the average of the other group, the two averages differ significantly from each other.

normal. Determinations were made by the method of Davis and Madsen ('41).

The total secretion of vitamin A is calculated as the product of the daily butterfat production and the A content. This is corrected for the average basal level which is determined in a similar manner. When this extra vitamin A secreted is divided by the vitamin A content of the supplement, the efficiency of secretion is obtained (table 4).

RESULTS

The results of the experiments on the butterfat of Guernsey cows are summarized in table 1 and those on the Holsteins are given in table 2. Table 3 reports the data on Guernsey cow 44 while table 4 shows the relative efficiency in elimination of the administered vitamin A in the milk. Figure 1 demonstrates the relationship between the levels of carotene in blood plasma and butterfat. The average carotene level in the blood plasma of Guernsey cows (eight in each group) was as follows: (a) basal period (7-2-41) 1360 ± 158 μg. per 100 cc. for six cows only; (b) vitamin A supplement period (8-16-41), controls, 762 ± 102, group I, 579 ± 73, group II, 412 ± 53; (c) post-vitamin A supplement period (10-6-41), 1060 ± 129, 1051 ± 119 and 969 ± 100 for the three groups, respectively. With the Holstein cows the average levels of plasma carotene of ten control cows and of eight cows in each of groups I and II were as follows: (a) basal period (7-21-41) 682 ± 58 for six cows only; (b) vitamin A supplement period (10-10-41) 783 ± 89, 423 ± 24 and 287 ± 38 for the controls, group I and group II, respectively; (c) post-vitamin A supplement period, 624 ± 67, 613 ± 40, and 522 ± 50 for the above groups, respectively. The ratio of mean difference to standard error of mean difference between the controls and experimental animals exceeds 3.00 with group II of Guernseys and both groups I and II of the Holsteins which indicates that the averages are statistically different. Moreover, the level of plasma carotene in group II of the Holsteins has a significantly lower value than that in group I.

The beta carotene and vitamin A per gram of butterfat in the milk of three groups of Holstein cows during a basal period followed by a vitamin A supplement period during which the basal diet was continued (control group) or when it was supplemented with different amounts of shark liver oil (groups I and II). No supplement was given to any groups during post-vitamin A supplement period.

SAMPLE FROM WEEK STARTING	WEEK OF PERIOD	VITAMIN A SUPPLEMENT IN I. U.		BETA CAROTENE IN MICROGRAMS			VITAMIN A IN INTERNATIONAL UNITS		
		Group I	Group II	Control Group	Group I	Group II	Control Group	Group I	Group II
				Basal Period					
July 17	1	0	0	3.59 ± 0.27	4.39 ± 0.41	4.24 ± 0.53	20.9 ± 1.5	28.2 ± 1.5	24.1 ± 1.0
July 24	2	0	0	4.28 ± 0.42	4.56 ± 0.55	4.33 ± 0.49	24.9 ± 1.6	27.4 ± 0.9	20.9 ± 1.5
Average				3.94	4.48	4.28	22.9	27.8	22.5
				Vitamin A Supplement Period					
Aug. 14	3	700,000	2,100,000	3.84 ± 0.43	3.87 ± 0.48	3.39 ± 0.31	36.9 ± 1.8 [1]	69.8 ± 2.2 [1] **11.72**	130.7 ± 5.4 **16.44**
Aug. 25	5	700,000	2,100,000	4.29 ± 0.31	2.99 ± 0.16 **3.61**	2.77 ± 0.24 **3.80**	33.9 ± 2.7 [1]	83.8 ± 3.3 [1] **11.59**	128.2 ± 6.8 **12.93**
Sept. 11	7	700,000	2,800,000	4.49 ± 0.38	2.75 ± 0.17 **4.24**	2.35 ± 0.14 **5.35**	44.7 ± 2.8	76.6 ± 3.7 **6.93**	174.4 ± 7.7 [1] **15.80**
Sept. 18	8	700,000	2,800,000	4.61 ± 0.39	2.65 ± 0.28 **4.08**	2.23 ± 0.15 **5.81**	47.5 ± 3.3	81.6 ± 3.1 **7.58**	167.9 ± 9.9 [1] **11.57**
Oct. 2	10	700,000	4,200,000	4.29 ± 0.35	2.88 ± 0.28 **3.13**	2.15 ± 0.20 **5.35**	40.7 ± 2.7	66.0 ± 3.4 **5.76**	280.4 ± 11.3 **20.65**
Oct. 9	11	700,000	4,200,000	4.96 ± 0.30	3.20 ± 0.21 **5.77**	2.50 ± 0.27 **6.15**	41.7 ± 2.8	76.5 ± 4.4 **6.69**	212.0 ± 14.1 **11.82**
				Post-Vitamin A Supplement Period					
Nov. 6 [2]	4	0	0	3.98 ± 0.33	2.85 ± 0.32 **2.45**	2.61 ± 0.19 **3.51**	31.0 ± 2.9	44.8 ± 2.4 **3.63**	49.0 ± 2.7 **4.50**
Nov. 26 [2]	7	0	0	4.91 ± 0.34	3.99 ± 0.45 **1.61**	3.92 ± 0.41 **1.83** [3]	21.4 ± 1.9	44.8 ± 2.0 **8.34**	44.6 ± 3.9 **5.38**
Dec. 16 [2]	10	0	0	3.61 ± 0.29	3.47 ± 0.30	3.36 ± 0.39	29.7 ± 2.0	32.9 ± 2.2 **1.07**	40.7 ± 1.5 **4.39**

Averages of ten experiments in all cases with control group and of eight experiments with groups I and II except where noted.

The figures for beta carotene and vitamin A include values for standard error of the mean calculated as follows: $\sqrt{\Sigma d^2/n} \,/\, \sqrt{n}$

The figures in bold face type are the quotients of the mean difference and the standard error of the mean difference. When this exceeds 3.00, the differences are considered statistically significant.

[1] One less sample than usual number in this group.
[2] Milk sample from single collection on this day.
[3] Significantly different from controls based on method II.

DISCUSSION

The depression in the carotene content of milk which results from the administration of large amounts of vitamin A in the form of shark liver oil has been shown to be attributable to vitamin A per se rather than to other components of this oil. Thus, the extent of the decrease in butter carotene of Guernsey cows (table 1) was equally as marked when the animals received 700,000 I. U. of vitamin A daily in the form of a vitamin A concentrate (1.4 cc.) as when a similar unitage of this vitamin was administered as 30 cc. of shark liver oil (25,000 I. U. per gram).

There seems to be some evidence also that within a certain range the extent of lowering of carotene is proportional to the amount of vitamin A administered. Thus, during the oil-supplement period the levels of this pigment are lower in every case in group II (which received 2,100,000 to 4,200,000 I. U. of vitamin A) than in group I (which received only 700,000 I. U. of vitamin A). Also the levels obtained in Guernsey cow 44 (table 3) are far lower than those reported when smaller amounts of the vitamin were given to other animals (table 1). However, as the carotene value reaches the minimum level, additional increases in vitamin A intake do not reduce further the carotene output. This is proved in both of the above series of tests where the vitamin A intake was greatly increased in the latter part of the test period without any corresponding decrease in provitamin A output.

This decreased carotene secretion is associated with a concomitant lowering in the carotene level of the blood plasma. Moreover, the depression in the level of plasma carotene is also greater when a larger intake of vitamin A occurs. This is evident both in the Holstein tests where the values for blood carotene in group II are significantly lower than those for group I as well as in the case of Guernsey cow 44 where the value is much lower than those for Guernsey groups I or II, both of which received a lower intake of vitamin A. In fact the minimum value of 100 µg. of carotene per 100 cc. of plasma

obtained on cow 44 is the lowest value recorded for any of the animals of either breed.

The decrease in carotene level cannot be the result of a lowered intake of this pigment. Although quantitative measurements were not made on the amount of alfalfa consumed,

TABLE 3

The beta carotene and vitamin A in the butterfat of Guernsey cow 44 before and after the administration of vitamin A in the form of shark liver oil.

EXPERIMENTAL REGIME	DATE OF START OF WEEK	WEEK OF PERIOD	VALUES PER GM. OF BUTTERFAT		BLOOD CAROTENE
			Beta carotene	Vitamin A	
	1941		*µg.*	*I. U.*	*µg./100 cc.*
Basal Period	June 25	1	10.47	29.0	...
	July 2	2	9.43	29.2	...
	July 16	4	9.47	18.4	...
Average			9.79	25.5	
Oil supplement period I	July 30	1	9.29	170.4	...
(2,650,000 I.U. daily	Aug. 6 [1]	2	6.85	208.8	...
starting July 30) [2]	Aug. 10 [1]	2	5.86	216.2	...
	Aug. 13 [1]	3	4.99	223.2	635
	Aug. 18 [1]	3	4.70	152.0	...
	Aug. 25	4	2.21	204.1	...
Oil supplement period II	Sept. 8	6	2.73	126.0	...
(5,300,000 I.U. daily	Sept. 15	7	2.75	147.2	...
starting Sept. 1) [2]	Sept. 29	9	2.80	221.2	...
	Oct. 9	11	2.28	178.4	100
Post-experimental	Nov. 6 [1]	4	3.61	32.9	...
period	Nov. 26 [1]	7	3.60	24.8	...
	Dec. 15 [1]	10	3.96	33.9	...

[1] Single sample for date specified rather than composite for week.

[2] Shark liver oil furnished by California Packing Corporation (Code F-148) which contained 94,800 I.U. per gram. During period I, 30 cc. was used daily, while during period II the quantity was increased to 60 cc. daily.

the amount of this feed eaten by a commercial herd of 156 Holstein and Guernsey cows fed shark liver oil for the production of a high vitamin A milk was not noticeably lowered. The low carotene level has been continuously observed in this commercial herd during a period of 9 months while this procedure has been employed.

The decrease might also be caused by a poorer absorption of carotene from the gastrointestinal tract in the presence of large quantities of vitamin A in the diet. We have no data on this point, although a study of it in chickens is in progress.

A more probable explanation would seem to be that an increased rate of destruction of carotene occurs in the tissues. The prolonged period required for the development of the maximum effect (4 weeks), as well as that during which it persists after the cessation of the feeding of the vitamin A supplement (7 to 10 weeks), would seem to indicate that the phenomenon may be related to the development of a new enzyme system. It has been suggested that the presence of large amounts of vitamin A in the tissues may cause the production of an enzyme capable of destroying this vitamin; such an enzyme would also be capable of altering carotene because of its close structural relationship to vitamin A.

The vitamin A content of the butterfat is directly proportional to the vitamin A administered. The average increase over the values of the control group in the Holstein cows receiving 700,000 I. U. for 6 different weeks (forty-six experiments) was 34.8 I. U.; when three times this dose was given, group II averaged 94.0 I. U. greater than the controls (sixteen tests); when the dose was four times this quantity, the mean increase was 125.0 I. U. (fourteen experiments); when six times the dose (4,200,000 I. U.) was administered, the augmentation amounted to 205 I. U. (sixteen experiments). The increase in vitamin A per gram of butterfat per million units fed for these four levels is 49.7, 44.7, 44.7, and 48.8 I. U., respectively, or an average of 47.0 I. U. However, due to the somewhat greater milk production in the group receiving 700,000 I. U. daily, the greatest efficiency in excretion of ingested vitamin A was noted here, i. e., 3.50%. In the Guernsey cows, the increase was somewhat less after the administration of shark liver oil at the level of 700,000 I. U. Here the butterfat contained on an average 37.0 I. U. more vitamin A per million units fed. This lower value in this species is partly to be traced to a longer period of saturation before the maximum

TABLE 4

Intake of vitamin A and its secretion in the milk.

BREED OF COWS	GROUP	WEEK OF PERIOD [1]	MILK PRODUCTION PER WEEK	BUTTERFAT PER WEEK	VITAMIN A SUPPLEMENT DAILY	VITAMIN A SECRETED DAILY		EFFICIENCY [2]
						Total	Corrected for basal	
			kilo	*gm.*	*I. U.*	*I. U.*	*I. U.*	%
Guernsey	I	0	95.4	3905	0	11,300		
		2	88.7	3608	700,000	16,600	5,400	0.78
		4	88.8	3645	700,000	17,900	7,200	1.03
		6	83.3	3496	700,000	24,900	13,600	1.93
	II	0	88.4	3759	0	10,900	....	....
		2	82.4	3481	700,000	24,600	13,700	1.98
		4	82.8	3713	700,000	25,300	14,400	1.95
		6	78.8	3321	700,000	31,200	20,300	2.91
Holstein	I	0	106.8	3430	0	13,700	....	....
		3	107.7	3490	700,000	34,200 [3]	21,000 [3]	2.86
		5	112.4	3730	700,000	45,700 [3]	32,200 [3]	4.60
		7	105.9	3455	700,000	37,900	24,200	3.48
		8	104.1	3449	700,000	40,300	26,600	3.80
		10	100.9	3306	700,000	31,300	17,600	2.76
		11	102.3	3477	700,000	37,900	24,200	3.47
	II	0	104.2	3501	0	10,700	....	....
		3	100.7	3367	2,100,000	62,900	52,200	2.60
		5	104.1	3683	2,100,000	64,300	53,600	2.57
		7 (2)	101.6	3216	2,800,000	80,100 [3]	69,400 [3]	2.50
		8 (3)	101.3	3126	2,800,000	74,900 [3]	64,200 [3]	2.34
		10 (1)	94.0	3249	4,200,000	129,000	118,300	2.81
		11 (2)	93.1	3688	4,200,000	111,300	100,600	2.39

[1] The figures in parentheses indicate the weeks on the increased oil supplement.

[2] Obtained by dividing the amount present in the milk (corrected for basal level) by the vitamin A supplement.

[3] Average of seven experiments only.

secretion into the milk, as the value obtained in the sixth week is higher than the average for the Holsteins receiving this dose. The increase in the Guernsey cows which received the same dose in the form of a vitamin A concentrate (group II) amounted to 48.6 I. U. or at a rate of 69.4 I. U. per million units fed.

The highest levels of vitamin A were obtained in the Holstein cows which received 4,200,000 I. U. of this vitamin daily. This averaged 280 I. U. per gram of butterfat for the first week, which is over twelve times the basal level and seven times the concurrent level of the control cows. Cow 56 in this group excreted butter containing 331 I. U. of vitamin A per gram. The maximum total vitamin A output was noted with cow 59 where 179,000 I. U. were excreted daily. The values obtained for the second week when the feeding continued at this level showed a marked drop in the level of vitamin A; however, this is in part accounted for in a marked increase in fat excreted so that the total vitamin A output is decreased to a considerably smaller degree. In the single test on Guernsey cow 44 at a correspondingly high level of intake, the maximum values of vitamin A in butterfat were somewhat lower.

Although the values are based on spectrophotometric determinations with the Bills-Wallenmeyer photometer, they are supported by bioassays made on three samples of Guernsey and three samples of Holstein butter. Moreover, practically identical values were found on eight samples from group II of the Holsteins showing the highest levels of vitamin A when carried out on the Beckman spectrophotometer and the Bills-Wallenmeyer photometer.

There is also a proportionality between blood carotene and the level of milk carotene (fig. 1). The comparative slopes of the curves are similar for Holsteins and Guernseys but at a more elevated level for the latter group. Considerably higher levels of blood carotene are associated with the secretion of a given amount of this component into the milk of the Holsteins as compared with that for the Guernseys.

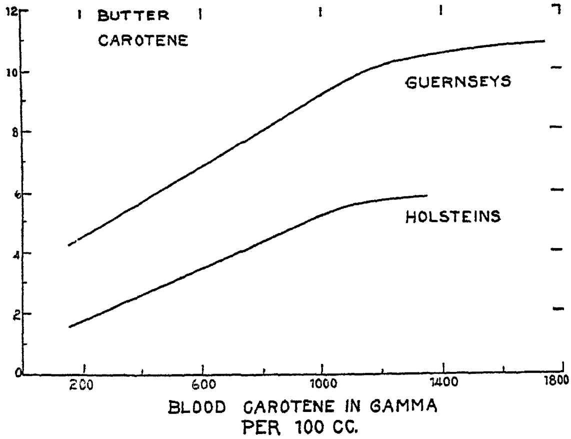

Fig. 1 The relation between the carotene in blood plasma and carotene concentration in micrograms in butterfat. The curves are plotted from average butter carotene values for each 100 μg. range of blood carotene.

SUMMARY

The decrease in milk carotene which follows the administration of shark liver oil appears to be caused by the vitamin A per se. A concomitant decrease in blood carotene also occurs when the milk carotene is lowered. The daily feeding of 2,100,000 I. U. of vitamin A as compared with 700,000 I. U. resulted in a greater decline in milk carotene; however, a further increase in dosage to 4,200,000 caused no further decline in carotene content of the milk. Approximately 4 weeks were required for the maximum lowering in carotene to develop while 7 to 10 weeks are required after the cessation of vitamin A supplement for the return of carotene secretion to normal.

The administration of vitamin A supplements is followed by an increase in vitamin A level of the butter in the Holsteins similar to that noted earlier with the Guernseys. The average increase in level of vitamin A was constant at approximately 47 units per gram butterfat for 1,000,000 units fed between an intake of 700,000 and 4,200,000 I. U. The maximum level of vitamin A noted was 331 I. U. per gram. The efficiency in secretion of ingested vitamin A is about 3%.

A proportionality exists between the levels of carotene in the blood and in the milk. Although the slopes of the curves for the Guernseys and the Holsteins are similar, that for the latter breed lies at a much lower level.

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VITAMIN CONTENT OF HONEYS[1]

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Honey is nectar of flowers brought by the worker bees to their hives and modified during the process of storing and ripening. Actually it is a plant product. Because the chief sources of vitamins for the animal world are plants, it is only natural that researches on the vitamin content of honeys were undertaken. Only the literature of the past 12 years is reviewed. Kifer and Munsell ('29) used white clover, buckwheat and honey-comb honey in their experiments and concluded that these three samples of honey produced in widely separated localities and representing extremes of color variation did not contain any detectable amount of either vitamins A, B, C or D. Vitamin B in this work refers to thiamine. Hoyle ('29) came to the same conclusion. Taylor and Nelson ('29), on the basis of their experiments, concluded that honey cannot serve as a source of vitamin E. Mathur ('30) found that honey contains no vitamins and even hastens the onset of polyneuritis in pigeons. Trautmann and Kirchhoff ('32), after a thorough investigation, concluded that the quantity of vitamin in honey is too minute to be discovered by the animal feeding experiments. Markuze ('35), employing bioassays, reported that two samples of honeys showed the presence of vitamin B_2. Vitamins A, B_1, C, and D were not found.

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Recently chemical determinations of vitamin C in honey have been made. Griebel ('38) reported 1.6–2.8 mg. ascorbic acid per gram of mint honey and 0.07–0.22 mg. per gram of some other samples. A biological assay on the mint honey (conducted by Scheunert at the request of Griebel) showed that 1 gm. a day completely protected some guinea pigs from scurvy. Kask ('38) demonstrated the presence of ascorbic acid in Esthonian honeys, the amount being from 0 to 20 (average 4.88) mg. per 100 gm. He also reported that the ascorbic acid content of honey decreases with age but that honey is a relatively stable medium for vitamin C. Werder and Antener ('38) determined the ascorbic acid content of nineteen samples of honey. The variation was from 1.1 to 14.6 mg. per 100 gm. Becker and Kardos ('39), employing microchemical methods, found from 31 to 89 mg. of ascorbic acid per 100 gm. of Castanea sativa honeys. On the basis of their animal tests, they concluded that the reducing component of honey is not vitamin C. However, Griebel and Hess ('39) proved the identity of the strong reducing substance in thyme and mint honeys with ascorbic acid. They reported 311.2 mg. and 102.6 mg. of this vitamin per 100 gm. of the respective honeys. Buckwheat honeys contained from 7.36 to 18.6 mg. ascorbic acid per 100 gm. Later ('40) the same authors investigated the ascorbic acid content of nectar of the abovementioned plants. A comparison of these data with those obtained on the honeys indicates that there is a considerable loss of vitamin C when the bees convert nectar into honey.

The development of the new microchemical and microbiological methods for the determination of vitamins has enabled research workers to determine the presence of even minute quantities of these materials in any substance. In view of the conflicting reports on the presence of vitamins in honey, we decided to investigate various honeys for their contents of thiamine, riboflavin, ascorbic acid, pyridoxine, pantothenic acid and nicotinic acid.

PROCEDURE

The honeys were obtained from beekeepers in various regions of Minnesota and from other localities of the United States. Samples of foreign honeys present in the University of Minnesota collection were also investigated for their vitamin content.

The methods used were: For thiamine, that of Hennessy and Cerecedo ('39); for riboflavin, that of Snell and Strong ('39); for ascorbic acid, Bessey ('38); for pyridoxine, Scudi ('41); pantothenic acid, Strong, Feeney and Earle ('41); and for nicotinic acid, the method of Melnick and Field ('40).

Where there was any question whether the method reveals the total content of any of these vitamins in honey, recovery of added vitamin was studied and found satisfactory. The most extensive study of this point was made for nicotinic acid. Each of five different samples of honey showing a range of nicotinic acid content from 12.8 to 52.0 mg. per cent was fortified with graded amounts of nicotinic acid, varying from 5 to 30 mg. per 100 gm. The recoveries ranged from 85% to 108.7% with a mean of 97.1%.

RESULTS

Table 1 gives a statistical analysis of the vitamin content found in twenty-nine samples of honeys from different localities of Minnesota. The main sources of these honeys were probably sweet and white clovers. Of course, there was more or less admixture of some other nectars typical for the state, such as basswood, black locust, mint, and fireweed, depending on the locality. To what extent they have influenced the vitamin content of the honeys cannot be estimated because the exact nectar composition of honeys was not known. From table 1 it is evident that the greatest variability was in the nicotinic acid content of the honeys investigated, with the ascorbic acid content following closely. The least variation was in the pyridoxine content of these honeys, riboflavin and thiamine being next.

Table 2 gives the vitamin content of thirty-one honeys from different regions of the United States and of seven honey samples from foreign countries. It is evident that there is a tremendous variation in the vitamin content of honeys from different sources and different regions. It is impossible to say whether these data are typical for the state and the source, because only one sample from each locality was investigated. However, the data in table 1 show that large variations may be expected even among samples having approximately the same nectar composition.

TABLE 1

Vitamin content of Minnesota honeys (per 100 gm.).

VITAMIN	MEAN	S. D.	C. V.
	µg.	*µg.*	%
Thiamine	5.5 ± .250	1.998	36.3
Riboflavin	61.0 ± 2.474	19.752	32.4
Pyridoxine	299.0 ± 11.457	91.471	30.6
Pantothenic acid	105.0 ± 5.54	44.227	42.12
	mg.	*mg.*	
Ascorbic acid	2.4 ± .155	1.237	51.5
Nicotinic acid	36.0 ± 3.486	27.831	77.3

Foreign honeys, as well as the honeys of the United States, show wide variations in their vitamin content. This is not surprising when one takes into account the difference in origin of those honeys and the different conditions under which the honey plants were growing. In no case, however, was the ascorbic acid content of the foreign honeys as high as has been reported by several European investigators. The question of whether the geological or climatic differences played any role in the variability of the vitamin content of these honeys remains to be answered after a thorough study of the samples from the same sources but collected in different regions.

All the natural honeys contain a certain amount of pollen grains and the type of honey can be determined by identifying the pollen (Armbruster, '34). Normal honeys have a somewhat cloudy appearance. Some beekeepers, desiring to in-

TABLE 2

Vitamin content of territorial United States and foreign honeys (per 100 gm.).

ORIGIN	NECTAR SOURCE	THIAMINE	RIBOFLAVIN	ASCORBIC ACID	PYRIDOXINE	PANTOTHENIC ACID	NICOTINIC ACID
		µg.	µg.	mg.	µg.	µg.	mg.
U. S. A.							
Washington	mixed	6.4	73	.6	300	87	78
Washington	clover and alfalfa	6.8	67	1.5	227	96	24
Washington	black locust	7.4	68	1.4	233	100	47
Washington	fireweed	8.2	81	4.1	397	56	13
Oregon	locust	4.3	35	.5	260	103	4
Oregon	alfalfa-sweet clover	4.3	36	1.3	430	175	92
Oregon	fireweed	2.2	62	1.4	260	87	84
Oregon	wild-buckwheat	4.3	56	2.8	250	180	16
California (1941)	star thistle	8.6	137	6.5	410	90	11
California (1941)	orange	8.6	35	2.5	210	150	16
California	orange	4.3	42	1.9	310	63	13
California	sage	3.0	36	5.4	320	56	4
California	star thistle	3.0	58	1.3	420	58	24
California	manzanita	7.1	..	2.5	280	..	8
Texas	cotton	2.1	58	.6	350	103	17
Texas	rattan	6.5	87	2.3	440	190	23
Long Island	mixed	6.5	46	2.0	240	155	26
Florida	tupelo (1934)	2.2	67	1.1	260	25	24
Florida	tupelo (1940)	4.3	58	2.1	250	118	44
New York (1936)	buckwheat	8.6	62	1.3	250	47	13
Tennessee	poplar	6.5	..	1.2	240	..	14
Tennessee	crimson clover	8.6	..	2.3	400	..	56
Hawaii	algaroba	8.6	46	2.3	250	50	32
Idaho	dandelion	6.4	87	2.5	267	192	11
Montana	clover	3.3	77	3.2	416	141	18
Montana	milkwort	9.1	..	2.9	250	..	17
Minnesota	mellon	7.1	36	2.6	400	156	58
Minnesota	mixed (1934)	8.6	46	2.8	480	31	86
Minnesota	mixed	7.1	53	3.6	260	25	23
Minnesota	mixed (1940)	9.1	40	.8	470	103	16
Minnesota	mixed (1941)	6.5	..	1.9	310	..	11
Foreign							
Greece	unknown	8.6	47	2.8	460	58	16
France	lavender	6.4	145	2.5	410	112	82
Czechia	linden	9.1	62	2.5	240	56	4
Azores	unknown	2.2	81	2.6	250	150	33
Haiti	logwood	3.0	65	1.5	280	50	15
Cuba	campanilla	2.2	58	1.3	420	56	67
Guatemala	coffee plant	7.1	62	2.5	250	60	94

crease the attractiveness of honey, have sought a method for its clarification. Such a method has been devised by Lothrop and Paine ('34). It consists of a thorough mixing of a small amount of rapidly filtering diatomaceous earth with the honey, heating to 140–160° F., and filtering. Paine and Lothrop ('36) reported that such honey is free from turbidity, has a brilliant appearance, and retains all of the natural components of honey, such as minerals, enzymes and flavoring substances.

Through the courtesy of Mr. G. P. Walton of the United States Department of Agriculture, we obtained a sample of honey which had been clarified by this process, together with a sample of the original, untreated honey. A study of these samples showed that the processing markedly reduced the vitamin contents of the honey, amounting to from one-third to almost one-half of the original values: thiamine decreasing from 8.4 to 5.4 μg.; riboflavin from 93 to 64; pyridoxine from 402 to 316; pantothenic acid from 176 to 97; ascorbic acid from 2.7 to 1.9 mg., and nicotinic acid from 87 to 46 mg. per 100 gm. of honey. Haydak and Palmer ('42) have found that bee bread (pollen stored by bees in combs) contains all the vitamins which have been shown to be present in honey. Therefore, the reduced vitamin content of the processed sample is no doubt partially due to the removal of the pollen. Moreover, some of the vitamins actually dissolved in the honey probably were adsorbed by the diatomaceous earth, decreasing the amount of vitamins still further. Therefore, the clarification, although it increases the attractiveness of honey, tends to reduce its food value and should be avoided. The study of the clarified honey also suggests that some of the variations in the vitamin content of different samples of honeys may be, at least in part, due to the variable amount of pollen present in honey.

SUMMARY AND CONCLUSIONS

Microchemical and microbiological determinations showed the presence in honey of thiamine, riboflavin, ascorbic acid, pyridoxine, pantothenic acid, and nicotinic acid. The amount

is extremely variable, depending probably on the source of honey and the number of pollen grains present in the product. The most pronounced variation found was in the nicotinic and ascorbic acid content.

Clarifying with diatomaceous earth diminishes the vitamin content of honey and therefore should be avoided.

Tables are presented showing a statistical study of the vitamin content of twenty-nine samples of Minnesota honeys, and the vitamin content of thirty-one individual samples of honey from various regions of territorial United States as well as seven samples of foreign honey.

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STUDIES ON THE HEMORRHAGIC SWEET CLOVER DISEASE

IX. THE EFFECT OF DIET AND VITAMIN K ON THE HYPOPROTHROMBINEMIA INDUCED BY 3,3′-METHYLENEBIS (4-HYDROXYCOUMARIN) IN THE RAT [1]

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ONE FIGURE

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The disease of cattle known in veterinary practice as the ''sweet clover disease'' arises from the ingestion of improperly cured sweet clover hay or silage (Schofield, '24). The syndrome is characterized by a hypoprothrombinemia resulting in a diminished clotting power of the blood, and the appearance of spontaneous hemorrhages (Roderick and Schalk, '31). Campbell and Link ('41) isolated the causative agent, and Stahmann, Huebner and Link ('41) showed it to be the dicoumarin 3,3′-methylenebis (4-hydroxycoumarin). After the hemorrhagic anticoagulant [2] became available

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Cooperative studies with the Division of Forage Crops and Diseases, U. S. D. A., Washington, D. C., through Dr. E. A. Hollowell. Personnel and supply assistance since July 1, 1938, in part through the Natural Science Research Project No. 65-1-53-2349 of the Federal Works Progress Administration (Madison), and since July 1, 1940, through special grants from the Graduate Research Committee of the University.

[2] The term ''anticoagulant'' is used in the general sense, that 3,3′-methylenebis (4-hydroxycoumarin) is an agent, which after action in vivo, impairs or prevents the coagulation of blood. It does not affect the clotting power when added to either blood or plasma in vitro (Campbell et al., '41, p. 12; Campbell and Link, '41, p. 36).

synthetically, its action could be studied in other species of animals, and characteristic hypoprothrombinemias were induced in rabbits, rats, guinea pigs, dogs (Overman et al., '42) and man (Bingham et al., '41; Butt et al., '41). In the rabbit it was observed that the extent of the response to the anticoagulant varied between fasted and fed animals. However, in view of the better status of our knowledge of the nutritional requirements of the rat, the latter species was selected for the present study.

METHODS

Two hundred sixty rats were used in these investigations. They were adults of both sexes, 4 to 14 months of age, either from our departmental colony or from the Sprague-Dawley Company. They were fed either of two basal rations, a stock ration or an artificial ration low in vitamin K (table 1).

TABLE 1
Composition of basal rations.

STOCK RATION		ARTIFICIAL RATION	
	%		%
Corn	40	Casein	18
Soybean meal	10	Yeast	8
Linseed oil meal	15	Salts (Wesson)	4
Alfalfa leaf meal	3	Cod liver oil	2
NaCl (iodized)	1	Dextrin	68
$Ca_3(PO_4)_2$	1		
Skim milk powder	20		
Butterfat	10		

Both rations are adequate for the growth and maintenance of rats.

The anticoagulant, 3,3′-methylenebis (4-hydroxycoumarin) was either incorporated into the basal ration and the mixture fed ad libitum, or the desired amount was added to the daily allotment of food. For convenience in routine feeding, the anticoagulant was "diluted" with cooked corn starch plus enough cottonseed oil (2%) to keep the mixture homogeneous.

The effect of 3,3′-methylenebis (4-hydroxycoumarin) on the clotting time of plasma was determined as follows: the

animals were fasted for 12 hours and then given the desired amount of the anticoagulant incorporated in 2 gm. of corn starch and oil. This was usually eaten within 15 minutes; no experiment was continued unless the entire supplement was consumed within an hour. Any scattered food was caught on papers placed under the cage, and the material returned to the food cup. Four hours after the consumption of the supplement the original basal ration was fed ad libitum.

Blood samples were taken at specific intervals after the administration of the anticoagulant. One and eight-tenths milliliter of blood was withdrawn by heart puncture under light ether anesthesia into a syringe containing 0.2 ml. of 0.1 M sodium oxalate. A recovery time of at least a week was allowed between determinations, and a rat was used as often as twenty times, thus permitting comparisons of the various factors under investigation upon the same animal.

The prothrombin time of the diluted plasma was determined by the procedure developed for the assay of hemorrhagic concentrates (Campbell et al., '41). Oxalated plasma was diluted with seven volumes of saline solution, and an aliquot added to a mixture of calcium chloride, saline solution, and thromboplastin from rabbit brain[3] under rigidly standardized conditions (Campbell et al., '41). All measurements were made by the same operator.

The prothrombin content of 12.5% plasma from normal rats was regarded as 100% of normal. The further dilution of such 12.5% plasma with saline solution and the determination of clotting times yielded a dilution curve in which the region of maximum shift in clotting time was the range 55–120 seconds. This corresponded to a concentration range of 85–20% of prothrombin.[4] Accordingly the doses of anticoagulant fed to

[3] Thromboplastin from rabbit brain functioned as well with rat plasma as a thromboplastin preparation from rat brain. Since the preparation from rabbit brain was available in greater quantities, it was used routinely.

[4] Since the absolute values (per cent prothrombin) vary according to the arbitrary concentration of plasma from which further dilutions are made (Overman et al., '42), our present results are expressed as clotting times of 12.5% plasma.

our rats were so chosen that the prothrombin times of the plasmas fell within this range.

EXPERIMENTAL

The response of rats to the continued administration of 3,3′-methylenebis (4-hydroxycoumarin). In general the response of rats to the continued administration of the anticoagulant was very similar to that reported for the rabbit, cow, or guinea pig (Schofield, '24; Roderick and Schalk, '31; Quick, '37) after the ingestion of spoiled sweet clover hay. The blood failed to clot and spontaneous hemorrhages appeared. These literally could be found in any part of the body with an adequate blood supply, and bleeding was either external or internal. In some animals large pools of blood accumulated in the subcutaneous regions or the abdominal or pulmonary cavities. In others, smaller and more numerous hemorrhages were found. Hemorrhage into the spinal canal was observed most frequently, and in older animals numerous clots formed in the skin. A small percentage of the animals showed diffuse hemorrhages in the thymus, the testes, or in restricted muscular areas, suggesting capillary weakness. Even in the absence of visible hemorrhage after continued administration, the animals looked ill, food intake and activity decreased, the hair became rough, and the animals felt cold to the touch. Some individuals developed a paralysis of the hind legs. The time of survival depended upon the dose. When 2 mg. of 3,3′-methylenebis (4-hydroxycoumarin) was added daily to the artificial ration, the animals died in an average of 14 days; when 1.5 mg. was given daily, survival averaged 29 days.

The specific detectable change induced in the blood by the anticoagulant was a marked reduction in prothrombin activity. However, neither the percentage of hemoglobin, the volume of red and white cells (hematocrit), nor the color of the plasma was altered except when severe hemorrhages had been proceeding for some time.

The effect of single doses of 3,3′-methylenebis (4-hydroxycoumarin) on clotting time. Plasma from rats fed the two basal diets was relatively uniform in its prothrombin activity; the 12.5% solutions clotted in 39 seconds, and 91% of 115 samples fell within the range 36–45 seconds. When 2.5 mg. of 3,3′-methylenebis (4-hydroxycoumarin) was given to rats on the artificial ration, the clotting time increased to 111 seconds, range 89–143. In a population of 400 rats fed the substance to date, all without exception developed a hypoprothrombinemia. An increase in the clotting time of the plasma could sometimes be detected 6 hours after the administration of the anticoagulant; it could be detected regularly after 8 hours, and the clotting time then increased until a maximum was reached 24 hours after the ingestion of the anticoagulant (fig. 1). The rate of recovery was roughly equivalent to the rate at which the hypoprothrombinemia developed originally. The extent and duration of the hypoprothrombinemia depended upon the amount of 3,3′-methylenebis (4-hydroxycoumarin) administered. When 1 mg. of the substance was fed, only a slight and occasional increase in clotting time resulted; when 5 mg. was fed, the plasma clotted so slowly that the significant range of measurement was exceeded (fig. 1). The response to the anticoagulant did not vary with repeated administration provided a recovery time of 1 week was allowed between doses.

Modifying effect of basal diet. Standard doses (2.5 mg.) of 3,3′-methylenebis (4-hydroxycoumarin) were given to rats fed either the artificial or the stock rations, and blood samples were taken in the usual manner 24 hours later. Other dietary additions were 30% of fat, 0.3% of choline, or the two together. While none of the diets altered the clotting time of the plasma in the absence of the anticoagulant, marked differences due to diet appeared in the degree of hypoprothrombinemia induced by 2.5 mg. of the substance. The 12.5% solutions of plasma from forty-four rats fed the anticoagulant plus the artificial ration clotted in 111 seconds, range 89 to 143; similar specimens of plasma from twenty-four rats fed

the anticoagulant plus the stock ration clotted in 58 seconds, range 40 to 82 (table 2). When animals were shifted from one diet to another, the effect of the previous diet usually did not hold over longer than 4 days, and it vanished in 7 days in 95% of the cases.

The additions of fat or choline to the basal diets did not materially alter the degree of hypoprothrombinemia induced by the anticoagulant (table 2) and only served to emphasize the much greater importance of the basal constituents of either

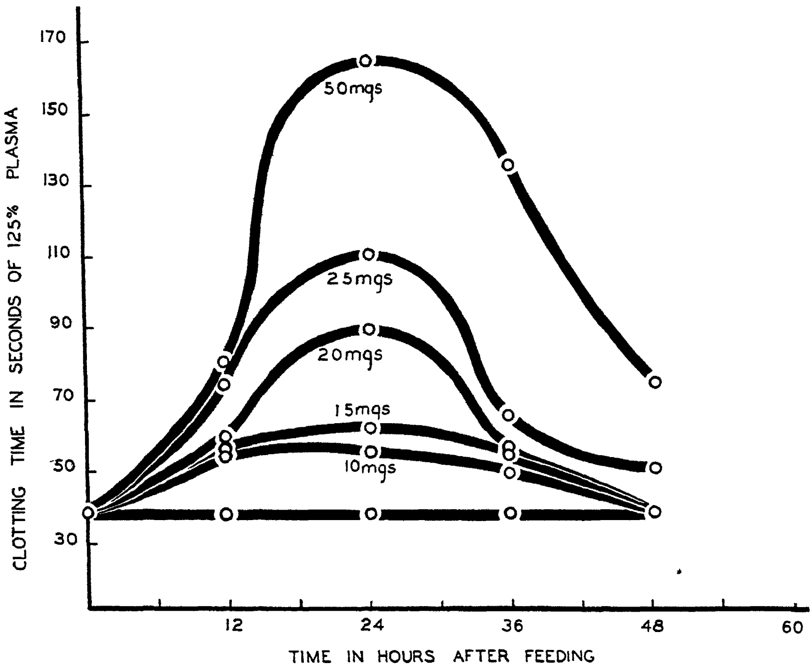

Fig. 1 The effect of 3,3′-methylenebis (4-hydroxycoumarin) on the prothrombin time of 12.5% rat plasma.

Each point represents the average of determinations on at least three animals. Some typical variations are as follows: 2.5 mg. of anticoagulant, 12 hours, average prothrombin time = 75 seconds (62–111) 22 determinations; 2.5 mg., 24 hours, prothrombin time = 111 seconds (89–143) 44 determinations; 2.5 mg., 36 hours prothrombin time = 82 seconds (65–105) 6 determinations; 2.5 mg., 48 hours, prothrombin time = 50 seconds (37–64) 6 determinations.

At this latter time, 48 hours, three of six animals showed a complete return to normal prothrombin time.

the stock or the artificial ration. However, the absorption of the anticoagulant from the digestive tract may have been retarded in the presence of large amounts of fat; a lesser degree of hypoprothrombinemia resulted after 24 hours than when the artificial diet was fed, and the rate of recovery from

TABLE 2

The effect of diet on the prothrombin time of 12.5% plasma from rats fed 2.5 mg. of 3,3'-methylenebis (4-hydroxycoumarin).

DIET	NO. OF DETERMINATIONS	ANTICOAGULANT FED	CLOTTING TIME IN SECONDS		
			Range	Average	Increase over normal
Artificial or stock	115	—	36–45	39	—
Artificial	44	+	89–143	111	72
Artificial + fat	23	+	64–200	99	60
Artificial + choline	5	+	110–168	135	96
Artificial + fat + choline	21	+	70–240	127	88
Stock	24	+	40–82	58	19
Stock + fat	7	+	45–66	58	19
Stock + choline	5	+	53–63	59	20
Stock + fat + choline	6	+	48–90	62	23
Potato diet	5	+	56–153	108	69
Low fat diet	4	+	83–117	97	58
Condensed milk [1]	22	+	79–132	101	62
Evaporated milk [2]	10	+	51–82	70	31
Stock ration minus butter fat	6	+	44–94	67	28
Artificial + 20% fish meal	3	+	44–84	65	26

[1] Borden's Eagle brand.
[2] Pet milk brand.

the condition was delayed appreciably. The anticoagulant induced a severe hypoprothrombinemia on several laboratory diets such as condensed milk, a low fat diet [5] and a potato diet (Quackenbush, Platz and Steenbock, '39).

Vitamin K. The artificial ration was substantially devoid of vitamin K whereas the stock ration contained the vitamin as a component of alfalfa. Accordingly the artificial ration was supplemented with 3% of alfalfa, with the petroleum

[5] Quackenbush, F. W., Kummerow, F. A., and Steenbock, H., unpublished.

ether extract of alfalfa equivalent to 3 or 20%, with the residue after extraction equivalent to 50%, or with an irradiated extract of alfalfa (10 hours to winter sunlight at −5 to −15°C.) equivalent to 20%. These diets were fed for periods of 3–10 days prior to the administration of the anticoagulant. The petroleum ether extract of alfalfa, as well as alfalfa itself, counteracted the prothrombin-depleting action of the anticoagulant, and this counter effect could be reduced by the irradiation of the extract. Fish meal as 20% of the diet likewise counteracted the anticoagulant (table 2). These observations were all consistent with the assumption that the active agent in these materials was vitamin K.

Large amounts of the vitamin, 2-methyl 1,4-naphthoquinone, were administered to rats either (a) incorporated into the artificial ration for 3 or for 7 days before the administration of the anticoagulant; (b) mixed with the supplement containing the anticoagulant; or (c) fed as late as 12 hours after the ingestion of the anticoagulant, i.e., after a reduction in the prothrombin activity of the plasma was already reflected by an appreciable increase in clotting time. The standard dose of 3,3′-methylenebis (4-hydroxycoumarin) fed was 2.5 mg. and blood samples were taken in the usual manner 24 hours later. The various dosages of vitamin K are indicated in table 3.

Vitamin K markedly decreased the extent and duration of the hypoprothrombinemia induced by the anticoagulant. The quantitative effects, however, varied with the mode of administration. Thus 0.2 mg. of 2-methyl 1,4-naphthoquinone fed daily for 3 or 7 days prior to the anticoagulant (total dose of vitamin = 0.6–1.4 mg.) was more effective than 2.5 mg. of vitamin fed with the anticoagulant. Partial protection against 3,3′-methylenebis (4-hydroxycoumarin) (clotting time = 55–75 seconds) was readily achieved by "moderate" amounts of the vitamin given in various ways (table 3). Complete protection (clotting time = 36–45 seconds) was achieved only when an enormous dose, 25 mg., of the vitamin was given. Even in the presence of these large doses, however, a transient

increase in clotting time was observed 12 hours after the administration of the anticoagulant, though recovery was complete in 24 hours. When lesser amounts of vitamin K were given, the clotting time, 12 hours after ingestion of the anticoagulant, was essentially the same as when no vitamin was given. However, during the second 12 hours, no increase in

TABLE 3

The effect of 2-methyl 1,4-naphthoquinone on the hypoprothrombinemia induced by feeding 3,3'-methylenebis (4-hydroxycoumarin).

AMOUNTS OF VITAMIN K AND HOW GIVEN	CLOTTING TIME OF 12.5% PLASMA			NO. OF RATS
	Artificial diet + vitamin K	Artificial diet	Grain diet	
	seconds	*seconds*	*seconds*	
Daily				
0.1 mg./day for 7 days	125 (114–136)	135	68	2
0.2 mg./day for 7 days	72 (69– 77)	121	45	3
0.5 mg./day for 7 days	77 (77– 77)	124	42	2
0.8 mg./day for 7 days	58 (53– 64)	115	58	4
0.2 mg./day for 3 days	61 (50– 71)	100	58	2
0.5 mg./day for 3 days	56 (50– 61)	126	60	4
8.0 mg./day for 3 days	54 (50– 64)	111	44	4
Singly				
1.5 mg. given with anticoagulant	127 (125–132)	123	62	3
2.5 mg. given with anticoagulant	86 (52–111)	114	50	6
25.0 mg. given with anticoagulant	40 (37– 44)	111	55	5
Singly				
2.5 mg. given 12 hours after anticoagulant	73 (62– 89)	119	58	5
5.0 mg. given 12 hours after anticoagulant	72 (59– 97)	119	59	7
25.0 mg. given 12 hours after anticoagulant	58 (54– 62)	125	57	3

clotting time occurred when vitamin K was present, whereas a very definite increase occurred in its absence. This suggested that the vitamin functioned primarily in stimulating recovery from the effects of the anticoagulant, possibly by increasing the rate of synthesis or activation of the prothrombin once the level in the blood had begun to fall.

Forms of vitamin K other than 2-methyl 1,4-naphthoquinone likewise counteracted the effects of the anticoagulant. Those tested include 2-methyl naphthohydroquinone 1,4 diacetate, vitamin K_1, and the water-soluble 2-methyl 1,4-naphthohydroquinone 3 sodium sulfonate.[6]

Survival experiments. The modifying effect of diet on the physiological potency of the anticoagulant was reflected not only in the clotting times of the blood plasma, but also in the periods of survival of the animals. In preliminary experiments 3,3′-methylenebis (4-hydroxycoumarin) was incorporated either into the artificial or the stock ration, and fed to rats ad libitum. The consumption of the anticoagulant averaged 2 mg. per day.

The average survival period for the rats on the artificial ration was 14 days. The range, 5 to 23 days, was wide, presumably because some of the animals failed to eat under the prolonged influence of the anticoagulant, and therefore lived longer than those which consumed the larger amounts of food and thus received more of the anticoagulant. However, only one of six rats on the stock diet died of spontaneous hemorrhages. Two others died from the effects of heart punctures on the sixtieth and seventy-fifth days, respectively, while the remaining three were in good health when the experiment was discontinued after 120 days of administration of the anticoagulant.

Similar results were observed when either 1.5 or 2.0 mg. of the anticoagulant was added daily to the artificial ration supplemented in various ways (table 4). The survival of the animals was not altered appreciably by the presence of either 0.3% choline, 3% wheat germ oil, or 30% of hydrogenated cottonseed oil (series III, table 4), nor by the ingestion of 30 mg. of ascorbic acid per day (series II, table 4). However, when 5 mg. of 2-methyl 1,4-naphthoquinone was fed daily to animals receiving 2 mg. of the anticoagulant daily, the

[6] We are indebted to Dr. Carl Nielsen of the Abbott Laboratories, North Chicago, Illinois, for this preparation.

animals survived the experimental period of 60 days. Similar animals receiving the anticoagulant but no vitamin K died in 14 days.

TABLE 4

The survival times of rats fed 3,3′-methylenebis (4-hydroxycoumarin) while on various basal rations.

SERIES	DIET	NO. OF RATS	MG. OF ANTI-COAGULANT DAILY	SURVIVAL IN DAYS
I	Stock	6	2.0 [1]	60 [2]
I	Artificial	6	2.0 [1]	14 (5–23)
II	Artificial	3	2.0 [1]	13 (5–27)
II	Artificial + ascorbic acid	3	2.0 [1]	13 (5–25)
III	Artificial	3	1.5	29 (16–43)
III	Artificial + 3% wheat germ oil	3	1.5	30 (24–38)
III	Artificial + 0.3% choline	3	1.5	24 (19–29)
III	Artificial + 30% fat	3	1.5	35 (18–66)
III	Stock	3	1.5	78 (no deaths)
IV	Artificial	3	2.0	14 (6–25)
IV	Artificial + 5 mg. vitamin K daily	3	2.0	60 (no deaths)

[1] Ad libitum consumption; estimated intake of anticoagulant.

[2] Only one spontaneous death in 60 days.

DISCUSSION

In the past, most studies on the physiological action of 3,3′-methylenebis (4-hydroxycoumarin) have been performed on the rabbit. Our present results indicate that the rat also readily responds to the compound, although certain differences between species are apparent. In the rat the hypoprothrombinemia induced by the anticoagulant is most severe after 24 hours, and recovery is usually complete after 48 hours. In the rabbit the maximum effect is attained after 48 hours or more, depending upon the dose administered (Overman et al., '42) and recovery may be delayed for as long as 6 days. Relatively large amounts of anticoagulant are tolerated by the rat. A single dose of 2.5 mg. lowers the prothrombin activity to 22% of normal in a 250-gm. rat, whereas this dosage produces a similar response in a susceptible rabbit weighing 2500 gm. (Overman et al., '42). Thus on the basis of body weight the susceptible rabbit is about ten times as sensitive

to the anticoagulant as the rat. Rats respond rather uniformly to the compound, whereas the individual variation between rabbits is large. Rabbits classified as resistant do not respond to a single oral feeding of 2.5 mg. of 3,3′-methylenebis (4-hydroxycoumarin), while in the extremely susceptible individual the action of 0.36 mg. (oral) is detectable.

The present experiments clearly indicate the need of the rat for vitamin K. Hitherto this has been demonstrated in rats with either bile fistulas or ligated bile ducts, but in normal rats the absence of the vitamin from the diet has only occasionally resulted in hemorrhagic tendencies (Greaves, '39; Dam and Glavind, '39). Many diets used routinely in the assay for other vitamins do not contain vitamin K, and it has been suggested that the organisms of the digestive tract furnish enough vitamin for the needs of the rat. However, when 3,3′-methylenebis (4-hydroxycoumarin) is ingested, the need for vitamin K appears to be increased so greatly that uniform responses to large doses of the vitamin are readily demonstrated. The mechanism is unknown by which the vitamin and the anticoagulant exert opposite effects but microorganisms do not appear to be involved. Both the anticoagulant and the vitamin function when given parenterally.

SUMMARY

1. The ingestion of 3,3′-methylenebis (4-hydroxycoumarin) induced a hypoprothrombinemia in rats. A single dose of 2.5 mg. to an adult rat lowered the prothrombin activity to 22% of normal in 24 hours, when maximum lowering was observed.

2. The maximum effectiveness of the anticoagulant was observed on rations low in vitamin K. Factors which counteracted the anticoagulant were present in a grain ration, in fish meal and in alfalfa. The factor in alfalfa could be extracted with petroleum ether.

3. Vitamin K counteracted the hypoprothrombinemia induced by the anticoagulant, whether given before the anti-

coagulant, with it, or 12 hours later. All forms of the vitamin studied were active: 2-methyl 1,4-naphthoquinone, 2-methyl 1,4-naphthohydroquinone diacetate, vitamin K_1 and the water-soluble 2-methyl 1,4-naphthohydroquinone 3 sodium sulfonate.

4. The continued administration of the anticoagulant resulted in severe spontaneous hemorrhages which caused the death of the animals. Their survival times could be prolonged by the ingestion of vitamin K but not by such dietary supplements as choline, ascorbic acid, wheat germ oil, hydrogenated fat, or condensed milk.

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THE RELATION OF THE SULPHUR AMINO ACIDS TO THE TOXICITY OF COBALT AND NICKEL IN THE RAT

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Possible interrelationships of cobalt, cysteine and choline are suggested by the observations of a number of investigators. Davis ('39) noted that choline prevents cobalt polycythemia in dogs; Michaelis and co-workers (Michaelis, '29; Michaelis and Barron, '29; Michaelis and Yamaguchi, '29; Michaelis and Schubert, '30; and Schubert, '31, '33) studied the formation and structure of complexes of cysteine with cobalt and nickel; and, Beeston and Channon ('36) and Mulford and Griffith ('42) have investigated the relation of choline to cystine. In view of the above findings a study was made of the utilization of sulphur amino acids in diets containing either added cobalt or nickel, with and without supplements of choline. The data reported in this paper clearly indicate the importance of the cobalt-cysteine relationship in rats because the injurious character of cobalt-containing food mixtures is readily decreased by administrations of either methionine, cystine, or cysteine, especially the latter. No evidence was found of the participation of choline in this mechanism. The concept that cysteine may act as a detoxicating agent for metals such as cobalt has not been reported previously as far as we have been able to determine, although Launoy ('34) found that cysteine is used in the detoxication of antimony, and Shinohara ('35) suggested the formation of a thiol complex in the detoxication of mercury.

EXPERIMENTAL

Young male rats, 20 to 26 days of age and 38 to 42 gm. in weight were used in all of the experiments. Diets were fed ad libitum and food consumption was measured, but these data are not included in this paper. The basal diets contained purified casein 12 to 30%, sucrose 37 to 55, lard 19, dried brewer's yeast[1] 6, agar 2, fortified fish liver oil 1, salt mixture 4 (Hawk and Oser, '31) and calcium carbonate 1%. Supplements of choline, sulphur amino acids and metallic salts either were added to the basal diets or administered separately as noted in the tables. Solutions to be injected were made as nearly isotonic as was possible. Animals on the stock diet were used in the acute toxicity experiments and were fasted for 4 hours before oral administration of supplements.

The effect of supplementary cystine and choline on the toxicity of cobalt and other metals. Groups 7, 13 and 19 (table 1) show the effect of 0.12% of $CoSO_4 \cdot 7H_2O$ on the rate of growth of rats on diets containing 12, 18 and 30% of casein. The greater protection on the higher protein diets was probably due to the increased level of sulphur amino acids because the effect of the cobalt was partially neutralized by a supplement of 0.5% of cystine (groups 9, 15 and 20). Groups 17, 14 and 16 demonstrate the effect on growth of increasing the cobalt concentration in the same basal diet. Groups 8, 9 and 10 illustrate the failure of choline to affect the toxicity of cobalt. In these experiments the injurious level of cobalt was approximately that reported by Stare and Elvehjem ('33), Josland ('36) and Josland and McNaught ('38). The data in table 1 show without question the efficacy of the cystine supplement although group 21 was the only group which grew normally on a diet containing cobalt. As will be shown later, cystine is very much less active than cysteine in the prevention of cobalt toxicity.

Table 2 shows the results of feeding diets containing equivalent amounts of cobalt, nickel, zinc, manganese, iron and copper. Of these elements only cobalt and copper showed evi-

[1] Anheuser-Busch, Strain G.

dence of marked toxicity which was partially prevented by cystine. The poor growth on the diets containing copper may have been due in part to loss of cystine as the insoluble cuprous-cysteine complex but apparently there was loss of

TABLE 1

The effect of supplementary cystine and choline chloride on the rate of growth of 40-gm. male rats on diets containing toxic levels of cobalt.

GROUP [1]	DIETARY CASEIN	SUPPLEMENT			AVERAGE TOTAL GAIN IN WEIGHT			
		Cobalt [2]	Cystine	Choline chloride	5 days	15 days	30 days	50 days
	%	%	%	%	gm.	gm.	gm.	gm.
1	12	0	0	0.05	12	34	69	112
2	12	0	0	0.30	12	37	76	122
3	12	0	0.5	0.05	15	44	90	140
4	12	0	0.5	0.30	13	39	97	146
5	12	0	0.5	1.00	12	43	89	140
6	12	0.12	0	0.05	—5	—5	..	...
7	12	0.12	0	0.30	—5	—6	..	...
8	12	0.12	0.5	0.05	12	35	68	92
9	12	0.12	0.5	0.30	11	33	70	92
10	12	0.12	0.5	1.00	10	26	50	75
11	18	0	0	0.3	17	42	84	...
12	18	0	0.3	0.3	16	51	97	152
13	18	0.12	0	0.3	0	7	10	...
14	18	0.12	0.3	0.3	11	24	35	40
15	18	0.12	0.5	0.3	12	39	71	103
16	18	0.24	0.3	0.3	0	8	..	...
17	18	0.06	0.3	0.3	13	40	75	113
18	18	0.06	1.0	0.3	19	58	98	135
19	30	0.12	0	0.3	5	25	26	...
20	30	0.12	0.5	0.3	15	46	83	...
21	30	0.06	0.5	0.3	12	50	97	142

[1] Six rats per group.

[2] Expressed as per cent of cobaltous sulphate ($CoSO_4 \cdot 7H_2O$).

other nutritive value because these food mixtures deteriorated rapidly on standing. This was also true if cuprous copper was used (unpublished experiments). Deterioration of the cobalt-containing food mixtures was not observed. The stimulation of growth resulting from a supplement of cystine in the

cystine-deficient 18% casein diet (Mulford and Griffith, '42) is also illustrated by the data in table 2.

The effect of supplementary methionine and cysteine on the toxicity of cobalt and the effect of intraperitoneal injections of the cobalt-cysteine (1:3) complex. Table 3 shows the comparative effects of cystine, cysteine and methionine on cobalt poisoning. Cysteine (groups 3 and 4) was more than twice as

TABLE 2

The effect of supplementary cystine on the rate of growth of 40-gm. male rats on diets[1] containing either added cobalt, nickel, zinc, manganese, iron or copper during a 30-day period.

GROUP [2]	SUPPLEMENT [3]	ADDED CYSTINE	AVERAGE TOTAL GAIN IN WEIGHT	GROUP [2]	SUPPLEMENT [3]	ADDED CYSTINE	AVERAGE TOTAL GAIN IN WEIGHT
		%	gm.			%	gm.
1	0.12% $CoSO_4 \cdot 7H_2O$	0	10	10	0.06% $MnSO_4$ (anhyd.)	0	95
2	0.12% $CoSO_4 \cdot 7H_2O$	0.3	35	11	0.06% $MnSO_4$ (anhyd.)	0.3	107
3	0.12% $CoSO_4 \cdot 7H_2O$	0.5	71	12	0.06% $MnSO_4$ (anhyd.)	0.5	107
4	0.11% $NiSO_4 \cdot 6H_2O$	0	78	13	0.11% $Fe_2(SO_4)_3$+Aq.	0	84
5	0.11% $NiSO_4 \cdot 6H_2O$	0.3	103	14	0.11% $Fe_2(SO_4)_3$+Aq.	0.3	100
6	0.11% $NiSO_4 \cdot 6H_2O$	0.5	97	15	0.11% $Fe_2(SO_4)_3$+Aq.	0.5	109
7	0.12% $ZnSO_4 \cdot 7H_2O$	0	90	16	0.11% $CuSO_4 \cdot 5H_2O$	0	30
8	0.12% $ZnSO_4 \cdot 7H_2O$	0.3	106	17	0.11% $CuSO_4 \cdot 5H_2O$	0.3	57
9	0.12% $ZnSO_4 \cdot 7H_2O$	0.5	113	18	0.11% $CuSO_4 \cdot 5H_2O$	0.5	72

[1] Basal diet: 18% casein + 0.3% choline chloride.

[2] Six rats per group.

[3] Approximately equivalent concentrations.

effective as cystine (group 2) or methionine (groups 6, 7 and 8). Group 5 shows the growth response following the oral administration of cysteine to rats after 20 days on the growth-inhibiting cobalt diet. The complex of cobalt and cysteine, while unquestionably less toxic than the cobalt alone, may in itself retain some toxicity. Lethal quantities of cobalt were injected intraperitoneally as the cobalt-cysteine complex (1:3) with partial inhibition of growth as the only apparent effect (groups 9 and 10). In these groups it was not evident whether

the inhibition of growth was due to the complex itself or whether some dissociation of the complex in the body liberated the much more toxic cobalt. The beneficial effect of supplements of cystine in the diets of rats receiving injections of the complex supported the latter suggestion (groups 11 and 12).

TABLE 3

The effect of the administration of cobalt with either cystine, cysteine or methionine on the rate of growth of 40-gm. male rats on diets containg 18% casein + 0.3% choline chloride.

GROUP [1]	SUPPLEMENT	ADDED COBALT [2]	ADDED CYSTINE	AVERAGE TOTAL GAIN IN WEIGHT	
				20 days	30 days
		%	%	gm.	gm.
1		0	0.3	70	97
2		0.12	0.3	30	35
3	0.39% cysteine hydrochloride	0.12	0	61	87
4	0.195% cysteine hydrochloride	0.12	0	41	58
5	20 mg. cysteine hydrochloride per os daily after 20th day	0.12	0	—4	17
6	0.31% dl-methionine	0.12	0	30	..
7	0.62% dl-methionine	0.12	0	44	..
8	1.24% dl-methionine	0.12	0	51	..
9 [3]	6.5 mg. $CoSO_4 \cdot 7H_2O$ as cobalt-cysteine complex	0	0	60	83
10 [3]	19.5 mg. $CoSO_4 \cdot 7H_2O$ as cobalt-cysteine complex	0	0	26	45
11 [3]	6.5 mg. $CoSO_4 \cdot 7H_2O$ as cobalt-cysteine complex	0	0.5	75	103
12 [3]	19.5 mg. $CoSO_4 \cdot 7H_2O$ as cobalt-cysteine complex	0	0.5	46	67

[1] Six rats per group.

[2] Expressed as per cent of cobaltous sulphate ($CoSO_4 \cdot 7H_2O$).

[3] Daily intraperitoneal injection of 1 or 3 cc. of a brown solution containing 1.7 mg. of cysteine hydrochloride and 0.9 mg. of $NaHCO_3$ per 1 mg. of $CoSO_4 \cdot 7H_2O$.

Toxicity and detoxication of cobalt by cysteine, glutathione (GSH), and methionine. High mortality resulted within 24 hours after the oral administration of 40 mg. and the intraperitoneal administration of 9 mg. of cobaltous sulphate (table 4, groups 3 and 7). The intraperitoneal injection of cysteine

was effective in permitting survival following either method of administration of the cobalt (groups 4 and 8). Furthermore, the cobalt-cysteine complex (1:3) was without lethal effect except at high levels (groups 5, 9 and 10). The complex was prepared as described by Schubert ('31) and contained 20 mg. of $CoSO_4 \cdot 7H_2O$, 34 mg. of cysteine hydrochloride and 17 mg. of $NaHCO_3$ per 3 cc. of solution. The unneutralized cobalt-cysteine complex gave a deep red solution which became brown after neutralization. On standing this solution darkened and deposited a black precipitate. Similar results followed the administration of either the red or brown solution. The solution in the peritoneal cavity was brown within 15 minutes after the separate injections of cobalt and of unneutralized cysteine hydrochloride. The injection of the brown complex was followed by its rapid absorption from the peritoneal cavity as was evidenced by the darkening of the urine, eyes, paws and subcutaneous tissues within 5 minutes after the administration. Reduced glutathione gave the same colored solutions with cobalt as did cysteine and the mixture of cobalt and glutathione was non-toxic (group 11).

Groups 12 to 16 (table 4) show the partial protection afforded by methionine against the toxicity of cobalt. The effect was less marked than that of cysteine, as was also the case in the growth experiments (table 3). It is not possible from the present data to determine whether methionine was effective either because of its conversion to cysteine or because of the formation of a cobalt-homocysteine complex.

The effect of cysteine on the toxicity of nickel. Michaelis and Barron ('29) have reported the similar behaviour of nickel and cobalt with respect to the formation of complexes with cysteine. Groups 17 to 23 (table 4) show that the toxicity of nickel is also decreased by the administration of cysteine. In agreement with Caujolle ('39) nickel appeared more toxic than cobalt insofar as mortality from single administration was concerned. However, as noted in table 2, nickel added directly to the ration proved much less toxic than an equivalent level of cobalt. Further work is needed to clarify these apparently contradictory results.

TABLE 4

The effect of cysteine[1] and of dl-methionine on the toxicity of cobalt and the effect of cysteine[1] on the toxicity of nickel in 40-gm. male rats.

GROUP	COBALT[2] USED	NICKEL[3] USED	PROCEDURE	NO. OF RATS	24 HOUR MORTALITY
	mg.	*mg.*			%
1	16	0	Per os	9	0
2	30	0	Per os	9	33
3	40	0	Per os	17	71
4	40	0	Cobalt per os + intraperitoneal injection of 34 mg. cysteine	16	0
5[4]	40	0	Cobalt-cysteine complex per os	21	0
6	6	0	Intraperitoneal injection	31	58
7	9	0	Intraperitoneal injection	27	89
8	16	0	Intraperitoneal injection of cobalt and 27 mg. cysteine	12	0
9[4]	20	0	Intraperitoneal injection of cobalt-cysteine complex	21	0
10[4]	40	0	Intraperitoneal injection of cobalt-cysteine complex	15	67
11[5]	20	0	Intraperitoneal injection of cobalt-glutathione complex	5	0
12	40	0	Cobalt and 64 mg. methionine per os	10	0
13	10	0	Intraperitoneal injection of cobalt + 64 mg. methionine per os	17	53
14	10	0	Intraperitoneal injection of cobalt + 26 mg. of methionine after 3 hourly injections of 12 mg. methionine	20	25
15	10	0	Two intraperitoneal injections of 5 mg. cobalt 5 hours apart + 32 mg. methionine per os one-half hour before and after first cobalt injection	14	0
16	10	0	Two intraperitoneal injections of 5 mg. cobalt 5 hours apart	5	100
17	0	20	Per os	28	64
18	0	20	Nickel per os + intraperitoneal injection of 34 mg. cysteine hydrochloride	12	50
19	0	20	Nickel per os + intraperitoneal injection of 17 mg. cysteine hydrochloride after 0 and 3½ hours	16	25
20	0	3	Intraperitoneal injection	7	14
21	0	6	Intraperitoneal injection	10	90
22	0	10	Intraperitoneal injection	6	86
23[6]	0	10	Intraperitoneal injection of nickel-cysteine complex	10	0

[1] Cysteine hydrochloride and one equivalent of $NaHCO_3$ were used.

[2] Expressed as mg. of $CoSO_4 \cdot 7H_2O$.

[3] Expressed as mg. of $NiSO_4 \cdot 6H_2O$.

[4] Brown solution of cobalt-cysteine (1: 3) complex containing 1.7 mg. cysteine hydrochloride and 0.9 mg. $NaHCO_3$ per mg. of $CoSO_4 \cdot 7H_2O$.

[5] Brown solution of cobalt-glutathione (1: 3) complex containing 3.33 mg. glutathione (GSH) and 1.5 mg. $NaHCO_3$ per mg. of $CoSO_4 \cdot 7H_2O$.

[6] Nickel-cysteine (1: 3) complex containing 1.7 mg. of cysteine hydrochloride and 0.9 mg. of $NaHCO_3$ per 1 mg. of $NiSO_4 \cdot 6H_2O$.

DISCUSSION

The above data suggest that the toxic effects of cobalt and nickel and possibly of copper may be largely, if not completely, neutralized by the presence in the diet of sufficient sulfhydryl compounds, such as, cysteine, reduced glutathione, or homocysteine (from methionine). These compounds are believed to detoxicate cobalt by the formation of less toxic complexes of the type described by Michaelis in the case of cobalt and cysteine. It may be that the principal effect in cobalt poisoning is that which results from the binding in complex formation of sulfhydryl compounds in the tissues. Indeed, it is conceivable that interference with cellular oxidations due to formation of such complexes, with glutathione for instance, may be the stimulus to the hematopoietic system which causes so-called cobalt polycythemia. Furthermore, these data suggest that consideration be given the possibility that dietary supplements of sulfhydryl compounds, or their precursors, may alter the outcome of studies of cobalt metabolism, and that dietary supplements of cobalt may alter the outcome of studies of sulfhydryl metabolism. In addition, the complex of cobalt and the sulfhydryl compound may itself be physiologically active.

It is significant that Barron and Barron ('36) and Davis ('40) have reported studies on the relation of ascorbic acid to cobalt polycythemia and have concluded that cobalt may bring about erythropoiesis by interference with some respiratory function of this vitamin. Although preliminary unpublished experiments have failed to demonstrate any influence of ascorbic acid on the detoxication of cobalt by cysteine in the rat, an interrelationship may exist between cobalt, ascorbic acid and a sulfhydryl compound, such as, glutathione. It is not possible at this time to coordinate the results of the present paper with the recognized role of cobalt as a dietary essential required for the prevention of anemia. The latter studies and the associated effects of an unisolated organic substance in liver extracts have been reviewed by Underwood ('40).

SUMMARY

1. The marked inhibition of growth of young rats due to 0.12% of cobaltous sulphate in an 18% casein diet is largely prevented by supplements of methionine, cystine or cysteine, especially the latter.

2. The high mortality due to either the oral or intraperitoneal administration of very toxic levels of either cobalt or nickel is prevented by the simultaneous and separate administration of cysteine.

3. The complex of cobalt and cysteine formed in vitro is relatively non-toxic.

4. Cobalt poisoning may be due to the fixation and loss of sulfhydryl compounds in tissues with resulting interference with oxidative mechanisms.

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STUDIES OF THE AVERAGE AMERICAN DIET

I. THIAMINE CONTENT

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Extended experimental studies of diets of restricted character have been carried out by numerous workers during the past three decades. The common purpose underlying these studies has been a determination, qualitatively and quantitatively, of the specific nutrients which are essential for good nutrition. Several species of animals have been used, the rat predominating. As a result, an extended series of vitamins, amino acids and minerals have come to be recognized as essentials and much has been learned of the consequences of an inadequacy of each. In such work it has been necessary to deal with simplified diets containing small numbers of food articles, each as rigidly controlled as possible and free from significant unknown constituents.

Some animal experimentation has been carried out with the more complicated diets which are prevailingly used for human nutrition (McCarrison, '36). The nutritional success attained with animals on such diets has often been meagre. Now that many of the necessary nutrients have been defined by the aid of experiments with simplified diets, it seems possible that the specific shortcomings of the more complex human diets can be detected by direct experiment.

For the purpose of such an attack, we have attempted to formulate a sequence of diets which contain all of the more important foodstuffs of the American dietary in proportions corresponding to the annual per capita consumption of each.

The principal basis of compilation has been the publication of Stiebeling and Phipard ('39). Their data have been critically verified and corrected by reference to figures from other sources.[1] Detailed discussion of the compilation will be reserved for later papers devoted to nutritional experiments with this sequence of diets. Suffice it to say here that all the major components recur in every diet, the variations in the successive members of the sequence being such as are introduced by seasonal variations in the availability of foodstuffs, etc. The staples are relatively few in number and these grossly predominate over the non-staples in their contributions to the total calories (table 3). The general make-up of the diets will be evident in illustrative examples furnished below. It is recognized that a sequence of diets comprising so many articles of food probably represents a greater variety than is usually available to the individual.

This paper deals with the thiamine contents of these diets. Later papers will deal with the contents of other vitamins and with the nutritional efficacy of the sequence of diets.

EXPERIMENTAL PART

I. Assay methods

For such an extended series of samples it was necessary to have an expeditious method and the thiochrome method as adopted for the analysis of cereal products (Frey, in press) was used after extended explorations of minor variants. The results were, on the whole, reassuring as to reproducibility. All thiochrome results reported in this paper represent the average of at least two independent determinations in which every step of the process including the extraction procedure was carried out in duplicate. Duplicate determinations rarely differ by more than 10% and in the exceptional cases probability of error in sampling was evident, for example, in meats streaked with fat and lean.

[1] Consultation with the service bureaus of the Department of Agriculture and Department of Commerce and correspondence with leading producers or trade associations of the principal food industries.

It was considered necessary to confirm the results of thiochrome assay of several representative foods by conducting animal assays of the identical samples. For this purpose the rat curative method was chosen.

Polyneuritis was developed by feeding 28-day-old rats on a commercial dog food [2] deprived of vitamin B_1 by the following procedure: 28 kilos of the meal contained in a rotating steel drum (55 gal.) was sprayed with 2800 cc. of water through a 2-inch opening in the head, and then with 2800 cc. of 5% solution of SO_2. Rotation was continued during spraying and for 6 hours thereafter to insure even distribution of the moisture. Except during spraying, the barrel was kept tightly closed for 6 days at a mean temperature of 30° C. with occasional rotation for an hour each day. The contents were then sprayed with 2700 cc. of 10% H_2O_2 and allowed to stand for 24 hours. The product was then dried at less than 50° C. and ground to a fine meal with addition of 270 gm. of linseed oil.

In early experiments with this method it was found that an excess of sulfur dioxide in the diet will prevent the cure of polyneuritis with normal doses of pure thiamine either by mouth or by injection. Destruction of excess sulfur dioxide with hydrogen peroxide was therefore adopted and each lot was analyzed for sulfur dioxide by iodometric titration of the distillate from a sample acidulated with excess phosphoric acid. Destruction of unsaturated fatty acids by hydrogen peroxide became evident through the development of a scaly tail condition of the rats during the development of polyneuritis. This was corrected by addition of linseed oil.

On such a diet well-defined polyneuritis develops more promptly and consistently than with any other diet with which we have had experience. Polyneuritis begins to appear in 3 weeks and is present in about 90% of the animals within 5 weeks. A few rats die without symptoms being observed and occasionally one continues to grow, exhibiting refection as verified by thiochrome analysis of feces. Refection in our

[2] Beacon dog food.

colony is not often accompanied by light-colored feces though we have frequently observed the latter. In seven rats out of ten which survive for as much as 24 hours after dosing polyneuritis is cured or significantly improved by oral or parenteral doses of 5 μg. of thiamine chloride. That quantity of a food required to produce an equal proportion of cures is assumed to contain 5 μg. of thiamine. Twenty rats or more are, on the average, required for the test of a foodstuff but a large proportion of them can be re-used two to four times. Foods of low potency cannot be tested by the method as polyneuritic rats will not ordinarily consume more than about 3 gm. of dry food solids.

COMPARISON OF RESULTS

Inspection of the thiochrome results revealed concordance with rat curative results except in the case of cooked pork, for which the thiochrome results were substantially lower. We were led to suppose that the low thiochrome results were due to failure of extraction. However, all attempts to improve extraction, even including a 12-hour digestion with normal sulfuric acid, failed completely to achieve the result. Experiments with proteolytic enzymes, pepsin, trypsin and papain were equally ineffective. We now surmise that thiamine pyrophosphate becomes partially bound by the cooking process through the reaction of the phosphoric acid group with the amino group of protein amino acids and that this linkage is split with great difficulty.

A similar binding of thiamine to protein can be effected by low temperature dehydration as indicated by the following and other similar experiments. The lean portion of three pork chops was ground, mixed and analyzed by the thiochrome method giving a result of 15.1 μg. per gram of fresh meat or 60.6 μg. per gram on a dry fat free basis. A portion of the meat was dehydrated by boiling with xylene under partial vacuum at $<60°$ C. till the distillate was water free. The remaining xylene was drained off of the meat, the adherent solvent evaporated at low temperature and the meat ground

to a powder. After such dehydration with xylene which effected a moisture and fat loss of 75.1%, analysis of the pork gave 35.6 µg. of thiamine per gram of dry fat free substance, indicating a reduction of 41% of the thiamine in the drying process. Rat curative assay, however, showed no loss. Apparent loss of thiamine may occur in the cooking process especially in flesh food submitted to a desiccating type of cooking. This has been ascertained by comparing thiochrome assays and rat curative assays of a number of foods prepared by customary cooking methods as shown in table 1. To ascertain how large an error is introduced by this factor in the

TABLE 1

Effect of cooking on thiochrome assays.

FOOD		THIAMINE Thio-chrome µg./gm.	THIAMINE Rat curative µg./gm.	BATCH NO.	THIAMINE Thio-chrome µg./gm.	THIAMINE Yeast fermen-tation [1] µg./gm.
Ham, smoked	Raw (42% H_2O)	11.8	11.9			
	Fried (15% H_2O)	8.5	11.9	30	0.79	0.79
Egg yolk	Raw	2.4	2.2	31	1.17	1.11
	Scrambled	2.2	2.2	32	1.20	1.34
Quaker oats	Raw, dry	5.3	5.0	33	1.11	1.47
	Cooked porridge	1.1	1.1	34	1.15	1.46
Pumpernickel bread		2.7	2.4	Average	1.08	1.23

[1] We are greatly indebted to Dr. C. N. Frey of the Fleischmann Research Laboratories for the yeast fermentation results.

thiochrome assay of cooked foods in general, we have also included in table 1 assays by the yeast fermentation method, with sulfite correction, on five mixed batches of food prepared as described in section IV of this paper.

It appears that thiochrome assays of certain cooked foods will tend to be slightly too low on account of this factor. However, it is a minor matter except in the case of pork, which is a major contributor of thiamine to the diet as a whole. In the case of smoked ham tabulated above, the apparent thermal loss in frying was 47% by thiochrome assay, but was actually only 32% by rat curative tests.

TABLE 2

Yield of thiamine per 100 gm. of foods as purchased.

FOOD	EDIBLE PORTION [1]	MODE OF COOKING	CHANGE IN WEIGHT IN COOKING	B_1 Raw edible portion	B_1 Cooked edible portion	B_1 Thermal loss	B_1 In total liquors [3]	B_1 Total loss [3]	YIELD OF THIAMINE IN MICROGRAMS Per 100 gm. raw edible portion [4]	YIELD OF THIAMINE IN MICROGRAMS Per 100 gm. as purchased [4]	REMARKS
	%		% E.P.[2]	µg./gm.	µg./gm.	%	µg.	%			
Cereal products											
White bread [5]	100	None	...	....	0.47	...	...	..	....	47	Bakers bread
Rye bread	100	None	...	....	1.70	...	...	..	....	170	Pumpernickel
Whole wheat bread	100	None	...	....	2.18	...	...	..	....	218	So labeled
Corn meal	100	Mush	+370	0.97	0.22	0	...	0	97	97	
Oatmeal	100	Porridge	+320	5.30	1.30	0	...	0	530	530	
Rice	100	Boiled	+237	0.84	0.10	44	15	61	33	33	Excessive discard of cooking waters
Dairy products											
Milk powder	100	None	...	2.76	. .	...	...	..	276	276	Used raw
Eggs	86	Boiled	−8	1.12	0.79	29	...	29	79	68	62 gm. per egg
	86	Scrambled	0	1.03	0.93	9	...	9	93	80	
Cheese, American	89	......	...	0.42	...	...	...	..	42	37	
Meats											
Bacon	100	Fried	−63	2.74	0.93	87	...	87	36	36	
Beef											
Round	96	Roast	−48	0.51	0.44	55	...	55	23	22	
Plate	63	Boiled	−31	0.33	0.18	15	10	62	28	18	Cooking water used
Chuck	75	Roast	−45	0.63	0.57	37	5.8	40	23	18	Juices used
Pork											
Ham	84	Fried	−27	11.81	8.52	47	...	47	622 [6]	520 [6]	
Loin chops	80	Baked	−48	14.84	8.76	70	...	70	455 [6]	364 [6]	
Shoulder	75	Roast	−47	10.51	8.75	55	...	55	466 [6]	304 [6]	
Head cheese	96	......	...	....	0.58	...	...	..	58	55	Delicatessen cooked, 63% water
Mutton											
Breast	70	Roast	−56	0.82	1.13	40	...	40	49	34	
Veal											
Chuck	58	Stewed	−40	0.73	0.21	65	3.4	82	26	15	Cooking water used
Chicken	73	Stewed	−20	0.80	0.22	43	18.8	76	46	33	Cooking water used
Fish, flounder	100	Fried	−33	0.84	0.75	40	...	40	50	50	
Halibut	84	Baked	−31	0.92	0.73	45	...	45	50	43	
Liver, beef	100	Fried	−28	2.28	2.62	17	...	17	189	189	
Vegetables, cooked											
Asparagus, canned	100	......	...	....	0.97	...	27.0	38	97	97	Can liquor used
Beans, canned	100	Baked	...	....	0.22	...	...	..	22	22	
Beans, lima, canned	100	Boiled	...	....	0.32	...	8.6	39	32	32	Can liquor used
Beans, snap	90	Boiled	+8	0.94	0.39	41	11.7	55	42	38	Cooking water discarded
	93	Boiled	+4	0.44	0.12	71	5.1	41	13	12	
Beets	55	Boiled	+3	0.25	0.02	80	17	92	2	1	Bought with tops intact. Cooking liquor discarded
Cabbage	81	Boiled	+4	0.37	0.18	17	3.5	49	19	15	Cooking water discarded
Carrots	44	Boiled	0	0.76	0.27	33	10.2	64	27	12	Bought with tops intact. Cooking water discarded
Corn, canned	100	......	...	....	0.26	...	...	..	26	26	
Eggplant	84	Baked	−34	0.53	0.72	10	...	10	47	40	
Onions	96	Boiled	−10	0.27	0.17	27	7.5	37	20	19	Cooking water used
Peas, canned	100	......	...	....	1.08	...	7.9	8	100	100	Liquors discarded
Peas, dried	100	Boiled	+190	6.1	1.10	48	...	48	318	318	Cooking water absorbed
Potatoes	76	Boiled	+8	0.84	0.50	19	143.0	36	55	41	Peeled raw. Cooking water discarded
Pumpkin, canned	100	......	...	....	0.16	...	...	..	16	16	
Sauerkraut	100	Steamed	−4	0.34	0.15	4	17.2	55	33	33	Cooking water used
Spinach	83	Boiled	+29	1.00	0.36	40	13.5	53	60	50	Cooking water used
Sweet potatoes	66	Baked	−19	1.09	0.41	75	...	75	27	18	Peeled after baking
Tomatoes, canned	100	......	...	....	0.54	...	...	..	54	54	Liquors used
Turnips	91	Boiled	+22	0.51	0.38	5.5	1.6	9	46	42	Cooking water discarded
Vegetables, raw											
Cabbage	97	......	...	0.37	...	...	...	..	37	36	
Carrots	47	......	...	0.76	...	...	...	..	76	36	
Lettuce	87	......	...	0.57	...	...	...	..	57	50	
Radishes	49	......	...	0.20	...	...	...	..	20	10	Circular no. 549 U. S Dept. Agriculture for edible portion per cent
Tomatoes	93	......	...	0.42	...	...	...	..	42	39	
Fruits, cooked											
Apples	75	Boiled	+8	0.19	0.12	29.5	0.3	32	13	10	Made into applesauce, including liquors
Prunes	81	Boiled	+53	0.39	0.25	0	1.6	4	37	30	Liquors used. Minimum water used in cooking
	80	Boiled		0.51	0.18	0	41.0	65	51	41	Liquors used. Much water used in cooking
Fruits, raw											
Apples	91	......	...	0.19	...	...	...	..	19	17	
Bananas	65	......	...	1.54	...	...	...	..	154	100	
Blackberries	100	......	...	0.22	...	...	...	..	22	22	
Cantaloup	53	......	...	0.48	...	...	...	..	48	25	
Grapes	92	......	...	0.67	...	...	...	..	67	62	
Honeydew melon	51	......	...	0.46	...	...	...	..	46	23	
Oranges	56	......	...	0.67	...	...	...	..	67	38	Drinkable juice
Peaches	65	......	...	0.14	...	...	...	..	14	9	
Raisins	100	......	...	1.06	...	...	...	..	106	106	
Strawberries	90	......	...	0.21	...	...	...	..	21	19	Raw, frozen
Watermelon	59	......	...	0.27	...	...	...	..	27	16	
Miscellaneous											
Peanuts	72	......	...	....	0.94	...	...	..	94	68	Circular no. 549 U. S. Dept. Agriculture for edible portion per cent
Chocolate	100	......	...	0.47	...	...	...	..	47	47	

[1] Per cent of "as purchased weight" after discarding inedible portions such as bones, cobs, tops, peelings, etc. Market practice as to vegetables varies considerably, tops being often included in whole or in part. Our market includes tops in as purchased weight.

[2] Calculated to represent the change in weight of edible portion only. The values are only approximate when the inedible portion is discarded after cooking as in case of bones in roasts, cobs from sweet corn and skins from potatoes baked whole.

[3] Total loss includes thermal loss plus B_1 in cooking liquors. If cooking liquors are consumed with the food the thermal loss is the only loss incurred.

[4] Includes thiamine in cooking waters unless otherwise indicated in "remarks" column.

[5] All breads used were bakers breads. The white bread was very generously supplied by Dr. C. N. Frey of Fleischmann Laboratories and was made according to the following formula, in grams: flour, Gold Medal, 100; water, 64; yeast, 2; sugar, 5; salt, 2; shortening, 3.5; diamalt, 1; dry skim milk, 5; arcady, 0.33.

[6] In using this table for calculating the thiamine contents of diets the lean pork yields should be increased by about 30% to account for unextractable thiamine in cooked meats.

II. The thiamine content of individual foodstuffs

In table 2 are given the results of the analyses of all articles of food entering into the sequence of diets. In each instance the cooking procedure used represented a common customary kitchen practice. Every effort has been made to include as "yield" all the thiamine which is destined to reach the mouth and no more.

The matter of cooking waters is particularly troublesome. Proportions of water used in cooking vary greatly from kitchen to kitchen. The extent to which cooking waters are discarded is also variable. We have endeavored to follow prevailing practice but have sought to enable the reader to ascertain the effect on the thiamine yield of deviations from our practice. If one wishes to include the cooking water with the food consumed in cases in which we have not done so, he may correct our "yield as purchased" values by adding the "B_1 in total liquors" or vice versa.

The values in table 2 do not represent the range of values which may be encountered for each of the foods. We trust they are typical values but have not been able to make numerous assays of each food for comparison. As shown in section IV, the use of these values for calculation has given totals for mixed diets in fair agreement with assays of the mixtures.

In the case of several meats, notably beef muscle, we recognize that our values are lower than recently reported by others (Waisman and Elvehjem, '41). Accordingly we have made curative trials on polyneuritic rats with five samples of beef. No cure of polyneuritis was achieved with less than 8 gm. of fresh lean raw beef; no improvement of symptoms was noted with less than 6 gm.; in two cases the curative dose was established at 10 gm. These results are in general accord with the older literature (Vedder and Clark, '12). We believe that Waisman and Elvehjem's samples were exceptional, or more probably their assays for the thiamine poor meats were in error due to the fact that large amounts of the basal diets of their animals were displaced by the test foods. Their assumption that all betterment of growth is due to thiamine in

the meat does not appear warranted when the test food reaches 10% or more of the weight of the total ration. For pork our results are in relatively good accord with theirs.

III. Various foods as sources of thiamine for the average American diet

The caloric contributions to diets by specific foods vary enormously and a staple food of low thiamine content may nevertheless contribute a major fraction of the total thiamine. By contrast many minor foods, though rich in thiamine, contribute only minor fractions of the total thiamine. Table 3 provides a presentation of the relative importance of various thiamine sources. It will be seen that the cereals and meats each supply about one-quarter of the total, dairy products and all vegetables supply about one-fifth each, and fruit yields about one-tenth of the total thiamine. It should be noted that the principal thiamine contributors are pork (other than bacon), milk and bread, while potatoes make a poor fourth.

If the white bread were replaced by whole wheat bread or "enriched" white bread containing the recommended minimum of 1.1 mg. per pound, the thiamine per 2500 calories of diet would be increased from 0.78 to about 1.28 mg. and the cereal contribution would be over 50% of the total.

IV. Thiamine content of mixed diets

The calculated thiamine yields of the foods of the average American diet as shown in table 2 were verified by the assay of several lots of the mixed diet as shown in table 4. For this purpose lots of the diet were made up by running each of the foods through a meat chopper and mixing all together in a power masticator. These lots did not contain every food listed in table 3. However, every lot contained white bread, sugar, milk powder, eggs, butter, lard and crisco, beef, pork, tomatoes, peas, lettuce, potatoes, raw carrots and raw cabbage in the proportions indicated in table 3. In addition, each lot also contained about fifteen other minor food articles so chosen

TABLE 3

Thiamine contributions from various foods to make a total of 2500 calories.

FOODS	GRAMS	CALORIES	CALORIES PER CENT OF TOTAL	THIAMINE μg.	THIAMINE Per cent of total
White bread (includes some sugar and fat)	260 [1]	679	27 3	122	16.9
Rye bread	4	9	0.4	7	1.0
Whole wheat bread	6	16	0.6	13	1.8
Corn meal	11	41	1 6	10	1.4
Oatmeal	4	17	0.7	20	2 8
Prepared breakfast cereals	6	20	0 8	1	0.1
Rice	5	16	0 6	2	0.3
Sugar (apart from bread)	97	384	15 3	0	0.0
Total cereal products	393	1182	47 3	175	24.3
Milk (as dry powder)	45	222	8 9	124	17 2
Eggs	34	48	1.9	23	3 2
Cheese	8	30	1 2	3	0.4
Butter	20	148	5 9	...	.
Total dairy products	107	448	17.9	150	20.8
Bacon (including fat pork)	7	43	1.7	3	0.4
Pork (other than bacon)	39	155	6 2	156	21.6 [5]
Mutton and lamb	6	16	0.6	2	0.3
Beef	46	104	4 2	7	1.0
Veal	7	10	0.4	1	0.1
Poultry	15	21	0 8	5	0.7
Fish	20	14	0 6	10	1.4
Liver and other organs	3	3	0 1	6	0.8
Total flesh	143	366	14.6	190	26.3
Fats and oils [2]	19	171	6.8	...	...
Asparagus (canned) [3]	4	1	...	4	0 6
Beans, baked (canned) [3]	7	7	0 3	2	0 3
Beans, lima (canned) [3]	2	2	.	1	0.1
Beans, snap	11	4	0 1	4	0.6
Beets	4	1	.	...	...
Cabbage, boiled (incl. cauliflower)	15	3	0 1	2	0.3
Cabbage, raw [4]	5	1	...	2	0.3
Carrots, boiled	10	3	0.1	1	0.1
Carrots, raw [4]	3	1	..	1	0.1
Corn (canned) [3]	13	13	0 5	3	0 4
Eggplant (incl. okra)	4	2	.	2	0 3
Onions	21	9	0.4	4	0.6
Lettuce	12	1	...	6	0.8
Parsnips and radishes	2	1	...	...	...
Peas (canned) [3]	13	7	0 3	13	1 8
Peas and beans, dried	7	24	1.0	22	3.1
Potatoes	138	100	4 0	57	7.9
Pumpkin (canned) [3]	7	3	0 1	1	0 1
Sauerkraut	3	1	...	1	0.1
Spinach	6	1	...	3	0.4
Sweet potato	20	22	0.9	4	0.6
Tomatoes (canned) [3]	18	4	0 2	10	1 4
Tomatoes, raw [4]	18	4	0 2	7	1.0
Turnips	4	1	.	2	0.3
Total vegetables	347	219	8.8	142	21.5
Apples, cooked	19	11	0 5	2	0.3
Apples, raw (incl. pears) [4]	37	21	0.8	6	0.8
Bananas	25	16	0.6	25	3 5
Blackberries (incl. other berries)	5	2	0 1	1	0.1
Cantaloup	7	1	. .	2	0.3
Grapes	5	3	0.1	3	0.4
Oranges and other citrus fruits	51	18	0.7	19	2.7
Peaches	10	5	0 2	1	0 1
Prunes, cooked	5	11	0 4	2	0.3
Raisins	2	6	0 2	2	0.3
Watermelon	15	2	0.1	2	0.3
Total fruits	181	96	3.9	65	9.1
Peanuts (incl. other nuts)	2	12	0.5	1	0.1
Chocolate	1	8	0.3	...	...
Total miscellaneous	3	20	0.8	1	0.1
Grand total	1193	2502	100.2	723 [6]	102.1

[1] Sufficient white bread was included in the diet to account for the total average white flour consumption. Part of this of course appears as other bakery goods, macaroni, etc., in customary human diets.

[2] This does not include fats contained in meats. A portion of the total shortening fats appears in the bread, that amount being deducted from the average consumption of fats and oils

[3] For convenience we used and analysed canned product but it should not be inferred that the product is not used in customary diets in other ways

[4] All foods other than fruits were cooked except when otherwise indicated. All fruits were raw except when otherwise specified.

[5] On account of thiochrome assay error this figure should be higher, perhaps about 27%. See footnote 6 to table 2.

[6] This would be increased to 775μg. if allowance is made for non-extractable thiamine in cooked lean pork.

as to preserve the balance of cereals, dairy products, meats, vegetables and fruits as shown in table 3. The selection of the fifteen minor articles was made so as to simulate the variety appearing in the home kitchen and to take advantage of seasonal variation in availability of foods in the market. All the articles in table 3 appeared in one lot or another of those shown in table 4.

TABLE 4

Thiamine content of mixed diets.

BATCH NO.	FAT	WATER	THIAMINE FOUND [1]	NON-FAT SOLIDS	GRAMS PER 2500 CALORIES	THIAMINE PER 2500 CALORIES	
						Found [2]	Calculated from table 2
			µg./gm.			µg.	µg.
18	3.5	54.6	0.66	41.8	1260	831	719
19	7.6	52.7	0.65	39.7	1100	716	650
20	4.9	55.3	0.46	39.8	1230	566	542
21	4.7	52.2	0.85	43.1	1160	989	699
22	4.2	54.7	0.69	41.1	1240	852	767
23	5.0	50.7	0.76	44.3	1180	894	732
24	7.8	53.3	0.71	38.9	1160	822	708
25	5.1	50.4	0.65	44.5	1180	766	677
26	3.8	55.7	0.43	40.5	1275	549	644
27	4.7	52.6	0.63	42.7	1170	740	775
28	4.2	55.5	0.67	40.3	1255	841	693
29	5.0	54.9	0.78	40.1	1220	950	621
30	8.3	21.3	0.79	70.4	683	540	537
31	10.8	17.8	1.17	71.5	653	764	649
32	12.1	12.7	1.20	75.2	610	732	676
33	12.5	12.9	1.11	74.6	608	675	760
34	11.5	15.7	1.15	72.7	634	729	740
					Average	762	681

[1] By thiochrome assay of mixture.
[2] Calculated on basis of 4 cal. per gram of non-fat solids and 9 cal. per gram fat.

CONCLUSIONS

The thiamine content of the average American diet, such as was consumed by the middle two-thirds or three-fourths of the population prior to the advent of enriched bread and flour, is about 0.8 mg. per 2500 calories. This is substantially lower than previously supposed from the results of computations such as those of Stiebeling and Phipard.

If the use of enriched flour and bread becomes universal, the average intake will be increased about 64% to about 1.3 mg. per 2500 calories.

Thiochrome assays of cooked flesh foods tend to give low values for thiamine on account of incomplete extraction. This error probably has a significant effect only on the thiamine contribution of lean pork to mixed diets. Desiccated flesh foods may give much too low thiochrome assay values.

The principal contributors of thiamine to prevailing diets are lean pork, bread and milk.

Tables are given to permit approximate calculation of thiamine yields of other diets as cooked and served.

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VITAMIN E, COD LIVER OIL AND MUSCULAR DYSTROPHY

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ONE FIGURE

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There is abundant evidence that tocopherol prevents and cures the various disorders produced in mammals and birds by diets containing cod liver oil. The disorders were first unwittingly produced by natural foods to which cod liver oil had been added to increase their content of vitamins A and D; hence the resulting pathology was ascribed to a toxic effect of cod liver oil. Such direct toxic action of cod liver oil was questioned on two occasions (Mattill, '38, '40) when the vulnerability of vitamin E to oxidation in the presence of unsaturated fatty acids undergoing autoxidation was discussed. In the muscular dystrophy of rabbits it was clearly shown that the dystrophy was a result of inadequate vitamin E, either because the diet contained none or because the vitamin E it was supposed to contain was destroyed before the animal got it. The production of identical disturbances in rabbits on vitamin E deficient synthetic diets containing no cod liver oil (Mackenzie, Mackenzie and McCollum, '41 a), finally disproved the theory of its direct toxic action.

The early experiments referred to were conducted [1] before tocopherol, as such, was obtainable and are belatedly described here because an unnecessary distinction is still being made between dystrophy induced by cod liver oil and

[1] Mr. T. U. Marron assisted in some of the early experiments.

dystrophy produced by a deficiency of vitamin E (Mackenzie, Mackenzie and McCollum, '41 b). Some additional observations are also recorded.

The basal synthetic diets were patterned after those used by Madsen, McCay and Maynard ('35) and contained the following ingredients in approximate percentages: cellulose, 20; casein, 15; sucrose, 10; starch, 35; lard, 3; cod liver oil, 3; yeast, 10; salt mixture, 4. When desired, vitamin E was added as 2 or 3% of wheat germ oil and, to prevent the fats from becoming rancid, 0.1 to 0.3% of a suitable stabilizer, such as hydroquinone, was sometimes introduced.

Young rabbits weighing 400 to 800 gm. were gradually shifted from rabbit chow to the basal diet. The typical effects of the various dietary modifications in a few animals, out of many, are shown in the accompanying graphs of their body weights.

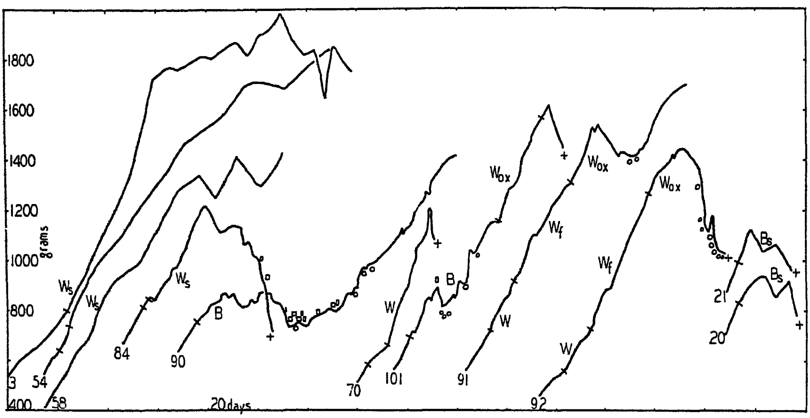

Fig. 1 B, basal diet; B_s, basal diet with added stabilizer; W, basal diet containing 3% of wheat germ oil; W_s, diet W containing stabilizer; W_{ox}, diet W whose three fat constituents (cod liver oil, lard, wheat germ oil) and yeast were mixed and kept in a current of warm air (60° C.) for 1 week before being added to the remaining components of the ration; W_f, diet W in which only the yeast had been exposed as in W_{ox}; after the incubation period as above, the yeast was washed free of the rancid fats, by ether, and fresh fats were used in making up the ration; the short lines crossing the curves mark the changes in diets; + indicates death from dystrophy; the small squares and rectangles above the curves indicate parenteral doses of dl-α-tocopherol acetate, the circles below the curves, oral doses.

On the basal diet containing a stabilizer (B_s 20 and 21) young rabbits died of dystrophy in about 3 weeks, often sooner, after showing a precipitous loss in weight and obvious external signs of dystrophy [2]; the presence of a stabilizer to delay rancidity was obviously of no avail in the absence of vitamin E. When the basal diet contained 2 or 3% of wheat germ oil without stabilizer (W, 70), the animals often did fairly well for some time but sooner or later dystrophy supervened. This is typical of many reports on the temporary effectiveness and ultimate inadequacy of wheat germ oil and other sources of vitamin E in alleviating the symptoms of its lack when unstabilized cod liver oil was present. The vitamin E content of such a diet depends on the freshness of the cod liver oil and on the conditions and duration of storage of the mixed diet. When in addition to wheat germ oil a stabilizer was included (W_s, 3, 54, 58, 84) the situation was greatly improved although even here, if the diet was made up in large lots, dystrophy might appear because the stabilizer lost its effectiveness during prolonged storage.

The remaining graphs further confirm the deleterious effects of rancidity. As described in the legend, diet W_{ox} contained the three fats (lard, cod liver oil, wheat germ oil) and yeast in a mixture in which rancidity was developing well before it was incorporated into the diet. In diet W_r only the yeast had been exposed to rancid fats. The behavior of animals 91 and 92 demonstrates the results. With a shift from the basal diet containing wheat germ oil (W) to diet W_r, growth continued, but on W_{ox} it soon ceased. Timely administration of α-tocopherol acetate [3] rescued animal 91 temporarily, but with animal 92 the administration was begun too late to prevent the fatal progress of dystrophy. Animal 101 was maintained on the basal diet by the help of tocopherol acetate and later succumbed on diet W_{ox}.

[2] In many cases the extent of muscle degeneration was determined by Dr. E. D. Warner in the Department of Pathology, to whom we are much indebted.

[3] α-Tocopherol acetate became available during the progress of these experiments and was generously supplied by Hoffman-LaRoche, Inc.

α-Tocopherol acetate was first administered intraperitoneally. The response of animal 90, as well as of other animals, demonstrated that the vitamin ester is less effective when given by this route than when given by mouth. More recently a similar difference has been noted with tocopherol itself in rabbits (Eppstein and Morgulis, '41) and in rats (Goettsch and Pappenheimer, '41).

The adequacy of diet W_f, whose yeast component was the only one to be exposed to autoxidizing fats, was suggestive but inadequate evidence that if any of the vitamin B factors are specifically concerned with the problem of dystrophy, they are not damaged by the oxidizing fats; the rancid conditions which favored the disappearance of vitamin E from the diet left the various B factors intact at least for the duration of the observations [4].

More recently the vitamin B factors appear to have been excluded from any complicity in muscular dystrophy (Mackenzie, Levine and McCollum, '40). That none of the unidentified B factors is concerned was demonstrated by the following experiment. Five young rabbits, which after 3 weeks on the basal diet containing yeast had not yet showed any signs of dystrophy, were shifted to a vitamin E-deficient diet like that quoted above, but containing 23% of casein and no yeast. Another group of five young animals was gradually transferred from rabbit chow to this yeast-free diet. Thereafter the following supplements were administered orally to both groups: α-tocopherol acetate, 25 mg. weekly in three doses; nicotinic acid, 5 mg., later 75 mg., weekly in three doses;

[4] The stability of some of the B factors under prolonged exposure to autoxidizing fats is not known. Doubtless all who have dealt with animals on E-deficient diets have occasionally encountered dermatitis in older animals and caudal necrosis in the new-born. Biotin appears to be easily destroyed by peroxides (Brown and du Vigneaud, '41). Both pantothenic acid and pyridoxine contain aliphatic hydroxyl groups, the latter also contains a phenolic hydroxyl group, as is the case in tocopherol; the dermatitis that is occasionally reported as alleviated by wheat germ oil (Richardson, Hogan and Itschner, '41) might have some such origin. The diminished liver stores of vitamin A in animals deprived of vitamin E for prolonged periods (Moore, '40; Bacharach, '40) can doubtless be explained in part on this basis.

choline hydrochloride, 100 mg. daily; thiamine hydrochloride, riboflavin, pyridoxine, 1.5 mg. of each, weekly in three doses; calcium pantothenate, 3.0 mg. weekly in three doses[5]. Two animals (in the first group) soon succumbed to dystrophy; the administration of tocopherol acetate was apparently begun too late to halt the onset and fatal termination of the disease. The remaining eight animals were maintained on the vitamin supplemented yeast-free diet for prolonged periods (25, 26, 36, 43, 83, 84, 91, 91 days) without the appearance of external symptoms of dystrophy. During this time, the rate of growth usually diminished and in some instances growth ceased altogether. Three animals died, at 36, 43 and 84 days, from various causes, but none with dystrophy.

The missing factors required for growth seemed to be present in fat-free liver powder[6] and in fresh grass[7] and dried grass[8]; when the diet of three animals was further supplemented by these materials at 83, 91 and 91 days, a rapid growth response was obtained in two of them, continuing for another 10 to 13 weeks. When the grass feeding was discontinued in the case of the third animal after 30 days it died in a few days with a liver pathology which was not further studied.

The various disorders produced in birds and animals by diets containing cod liver oil can all be explained as due to lack of vitamin E; in the case of diets known to contain vitamin E originally, this lack is the result of its oxidation which, like that of carotene (Sumner and Sumner, '40) and vitamin A (Dyme, Nelson, Howe and Nelson, '41) is coupled with the oxidation of fatty acids. In this association vitamin E is more readily oxidized than vitamin A. All of the observations recorded in the literature, many of them very confusing, can be reconciled on the basis of a disappearance of vitamin E from the rations used, either before or after their ingestion,

[5] The last four of these were kindly furnished by Merck and Co.

[6] Kindly supplied by Wilson Laboratories.

[7] Prepared as described by Foster, Jones, Russell and Dorfman ('40).

[8] Cerophyl. Courtesy of Dr. Kohler, Cerophyl Laboratories.

the rate of this disappearance being dependent on the character and rate of oxidation of the accompanying unsaturated fats. This is made particularly clear by the innocuous character of hydrogenated cod liver oil (McCay, Paul and Maynard, '37) and by the observations of Mackenzie et al. ('41 a), that 3 mg. of tocopherol, fed separately, prevented dystrophy in rabbits on a diet containing lard, whereas this amount of tocopherol did not suffice to prevent dystrophy when the diet contained cod liver oil or when cod liver was administered separately shortly following the tocopherol. The induction period of lard is much longer than that of cod liver oil; hydrogenation greatly prolongs the induction period of cod liver oil.

As there has long been no reason for speaking of a salt ophthalmia, so there is now no reason for distinguishing between a dystrophy produced by lack of vitamin E and a cod liver oil-induced dystrophy.

SUMMARY

Evidence is presented to show that no distinction need be made between a cod-liver oil-induced muscular dystrophy in rabbits and the nutritional muscular dystrophy produced by lack of vitamin E. None of the members of the vitamin B complex appears to be concerned with nutritional muscular dystrophy.

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THE VITAMIN E CONTENT OF CERTAIN VARIETIES OF WHEAT, CORN, GRASSES AND LEGUMES AS DETERMINED BY RAT ASSAY

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ONE FIGURE

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The importance of accurate information on the vitamin E content of foods and feedstuffs has increased manyfold with recent developments in the physiological aspects of the chemical substances possessing vitamin E activity. The needs of farm animals for vitamin E still remain obscure but it is apparent that a marked clarification of the problem will result from better knowledge of the vitamin content of animal feeds. Animal assays are tedious and time-consuming. Without a definite standard of reference, results obtained by various laboratories, using methods perhaps suited to the particular product in question but not universally applicable, have often lacked the desired precision and uniformity. The availability of a synthetic source of vitamin E in the form of alpha-tocopherol now appears to offer a means of expressing the vitamin E potency of feedstuffs on a more uniform basis. In the present report, some experiences are described with an assay procedure in which alpha-tocopherol has been used as a standard and the technique applied to the assay of a number of feedstuffs including the cereal grains and green and dried grasses.

The general plan for biological assay of vitamin E was developed by Evans and Burr ('27) and described more recently by Palmer ('37). The latter author points out some of the disadvantages of the method, among which is the fact that there is no definite answer to the question of how late in the gestation period vitamin E may be fed with assurance that it will exert a physiological effect. Mason ('39) reported results which apply to this aspect of the subject, but a further problem is presented in the assay of feedstuffs which are (a) low in vitamin E content, (b) bulky or unpalatable to rats, and (c) must be fed over a considerable period of time to secure the desired consumption. In view of these facts it appeared desirable, in the course of establishing an assay procedure, to obtain data showing the effect of feeding single doses of concentrated vitamin E supplements on different days as compared to continued feeding for definite intervals during the gestation period.

EXPERIMENTAL

The procedure followed is essentially the same as described by the authors just mentioned. The principal exception has been the introduction of alpha-tocopherol as a reference standard in order to express vitamin E content as weight of alpha-tocopherol per 100 gm. of feedstuff.

Groups of female rats at the age of 28 days were placed in cages with raised screen bottoms and fed the basal ration, consisting of dextrin 71.5%, casein (alcohol extracted) 18%, salt mixture 3%, brewers' yeast 7%, fortified cod-liver oil 0.5%. The fat content of this diet was less than generally used in vitamin E-free diets but the purpose was to avoid rancidity and deterioration and the possibility of including a source of vitamin E. Fortified cod-liver oil was added just prior to feeding. Preliminary experiments with the diet showed it to be fully as satisfactory as the high-fat diets for all purposes, and superior from the point of view noted above. At the age of 110 to 120 days the animals were mated by

placing a normal male rat in the cage overnight. Vaginal smears, taken with a dropping bottle syringe filled with water were examined microscopically for sperm the next morning. The females showing no sperm were left in the mating cage and examined daily until positive mating occurred. The time required for positive mating was frequently much longer than 5 days or the normal length of the estrous cycle. However, animals showing the erythrocyte sign were rarely found in the mating cages, indicating that the method of examining the female for sperm after being mated over a 16-hour period was reliable.

Animals showing positive mating were placed in individual cages and fed the desired assay material apart from the basal diet, the method of feeding used depending on the nature of the supplement fed. The usual procedure of examination for erythrocyte sign and daily weighing after the thirteenth day was followed to determine pregnancy and the event of either resorption or birth of a litter.

The use of virgin females for vitamin E assay has been recommended by Mason and Bryan ('38) and Bacharach et al. ('37). The increased requirements of female rats for vitamin E with age has been demonstrated by Evans and Emerson ('39) and Emerson and Evans ('39 a, b). Barrie ('38) has discussed both uterine degeneration due to vitamin E deficiency and resorption with its possible effects on vitamin E assay. In the assays reported here, preliminary attempts to standardize animals indicated that females of several classes with relation to gestation history could be used without sacrificing accuracy as follows: (1) virgin females which have been on the vitamin E-deficient diet 70 to 90 days; (2) females which have resorbed on the first gestation when fed an insufficient vitamin E supplement; and (3) females which have given birth to a litter on the first gestation when fed a vitamin E supplement, followed by a resorption. The animals of different classes were equally distributed on each supplement so as to avoid inaccuracy that might result from differences in previous reproductive history.

METHODS OF FEEDING VITAMIN E SUPPLEMENTS

The cereal grains were ground and the total amount to be fed weighed into an ordinary feed cup, separate from the basal diet, and the entire feeding given to the animal to be eaten ad libitum.

The green grass was chopped into approximately quarter-inch lengths, weighed, and put into a container which was kept in the refrigerator; small portions were fed daily to avoid drying.

In order to insure complete consumption of the dried grass supplements, they were first ground to pass a 40-mesh screen and mixed with casein and sugar as follows: dried grass, 25%; casein (alcohol extracted), 12.5%; and white cane sugar, 62.5%. The required amount of the mixture to supply the proper quantity of grass was placed in a feed cup and in most cases was consumed within a few days. Occasionally animals required as long as 10 days to consume the grass mixture, but when consumption was unusually slow and if more than 1 or 2 gm. of the grass mixture remained unconsumed after 10 days, the animal was discarded.

The alpha-tocopherol dissolved in ethyl laurate, and the wheat germ oil were dropped from a calibrated syringe directly into the animal's mouth.

EXPERIMENTAL RESULTS

Effectiveness of vitamin E fed at various stages of the gestation period

Two experiments were conducted in order to establish as nearly as possible the maximum period of time over which the feeding of a vitamin E supplement for assay could be extended without loss in the possible physiological effect of the total vitamin E. Wheat germ oil as a source of the vitamin was fed to one series of animals, and alpha-tocopherol to another. In the selection of the time intervals for feeding these supplements, the day on which sperm appeared in the smear was considered the first day. Certain groups of animals were given

the complete dose on the second day; others, on the seventh or the twelfth day. In still other groups the dose was divided into 10 or 20 equal feedings fed over as many days.

As shown in table 1 the feeding of 125, 250 and 500 mg. of wheat germ oil in a single dose on the second day was somewhat less effective than the same amount distributed over the first 10 days of the gestation period. Likewise there is a similar but less sharply defined distinction in the case of animals fed 1 mg. of alpha-tocopherol as a single dose on the second

TABLE 1

Effectiveness of varying doses of vitamin E with relation to different intervals in the gestation period as determined by the feeding of wheat germ oil and alpha-tocopherol.

INTERVAL	RATS FED WHEAT GERM OIL					RATS FED ALPHA-TOCOPHEROL				
	Dose	Gestations		Young		Dose	Gestations		Young	
		Number	Producing litters	Live	Dead		Number	Producing litters	Live	Dead
days	*mg.*		*%*	*no*	*no.*	*mg.*		*%*	*no.*	*no.*
2nd	125	8	12.5	3	0	0.66	8	37.5	6	2
2nd	250	12	58.3	8	8	1.00	17	70.6	53	4
2nd	500	14	78.6	24	13	2.16	6	100.0	27	1
7th	250	8	37.5	2	2	1.00	8	62.5	20	0
7th	500	11	45.5	18	1	2.00	8	62.5	19	4
12th	500	9	11.1	0	1	2.00	6	33.3	0	4
1st 10	12.5	6	33.3	2	2	0.10	14	85.7	52	5
1st 20	12.5	7	57.1	10	3	0.05	12	75.0	26	5
1st 10	25.0	7	85.7	9	4					
1st 10	50.0	5	100.0	17	9					

day and as 0.1-mg. daily doses for 10 days. Delay in feeding the test material until the seventh day still permits reasonably effective protection against resorption, while by the twelfth day only a small percentage of females give birth to young and these are usually dead. Distribution of the test feeding over a 20-day period results in some loss of effectiveness as compared to a 10-day period. Also of interest is the indication of the quantitative requirement of synthetic alpha-tocopherol for reproduction in the rat. It is evident that 1 mg. is not suf-

ficient, but a 2 mg. dose is probably a dependable level. This is in close agreement with results reported by Goettsch and Pappenheimer ('41). The use of wheat germ oil entailed some difficulties due to slow but unmistakable loss in vitamin E potency. From spectrophotometric examinations the alpha-tocopherol was found to remain stable throughout the test period.

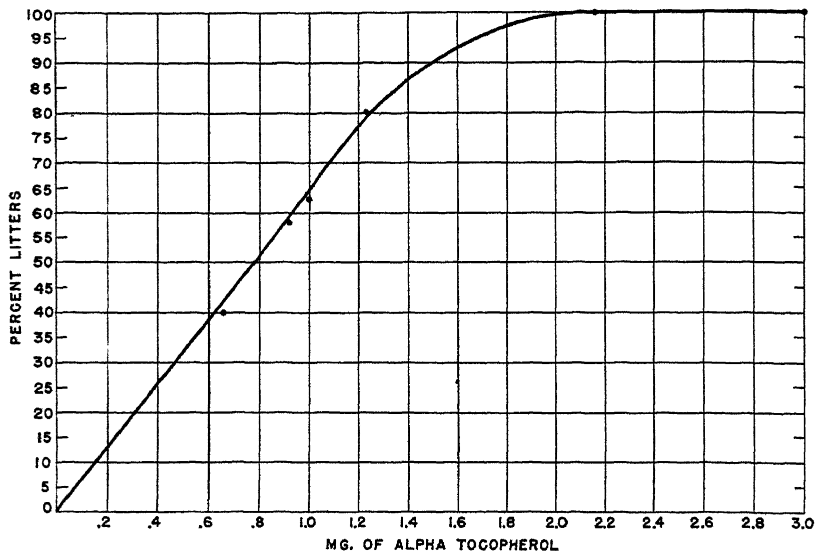

Fig. 1 Reference curve for vitamin E assay showing percentages of litters obtained from pregnant females fed alpha-tocopherol at different levels.

In the subsequent work, alpha-tocopherol was used as a reference substance in the assay of the grains and grasses which were studied. For this purpose, the alpha-tocopherol was fed at various levels in order to establish a reference curve as shown in figure 1.

Data on which the first four or most critical points of the curve were based comprise the results from thirty-five gestations. Each dot along the curve represents the average percent of litters obtained from feeding the indicated amount of alpha-tocopherol. The results show a high correlation of percent of

litters with amounts of alpha-tocopherol fed. Obviously in the establishing of such a curve of reference for use with a given series of test samples, the percentage value for a particular level of alpha-tocopherol may depart considerably from the normal pattern with a change in the results from one or two animals. Such an instance is illustrated in the result given in table 1 for the feeding of 1 mg. of alpha-tocopherol on the second day of gestation. The value given is 70.6%. Had one less female produced a litter, the resulting average value for the group would have been approximately 65% which would have fallen on the curve as drawn. It is obvious that new curves of reference should be established with each succeeding series of assays in order to avoid errors due to changes in response of the animals.

It is apparent that values for vitamin E content of supplements which have been fed at measured levels can be read easily from such a curve. Any experimental error in the curve, of course, will be reflected in the computed values, but the method has the obvious advantage that results obtained even under different experimental conditions may be more accurately compared. The feedings were all made on the second day. In the light of the data presented in table 1, some increase in accuracy might be achieved by distributing the alpha-tocopherol feedings over a period equal to that required for the ingestion of the feed to be tested and preferably on approximately a 10-day basis when the material under test is low or only moderately rich in vitamin E.

Vitamin E content of varieties of wheat and corn

Samples of five varieties of wheat grown on the Arlington Farm of the Bureau of Plant Industry, United States Department of Agriculture, were compared with a group of samples representing four of the same varieties from the Ohio Agricultural Experiment Station, Wooster, Ohio. Six varieties of corn, three yellow and three white, grown on the Arlington Farm were also assayed. Table 2 shows the results obtained

on these samples expressed as milligrams of alpha-tocopherol per 100 gm. of grain and as grams of grain necessary to give litters in four out of five pregnancies. These calculated figures represent results from gestations of 283 animals, an average of almost eighteen animals for each sample. In most cases the samples were fed at three different levels.

The method followed in calculating the vitamin E content from the curve shown in figure 1 may be illustrated by the following example. The wheat sample of the Poole variety from the Arlington Farm was fed at three levels, 20, 30, and 50 gm. The 20-gm. level gave three litters and five resorptions or 37.5% litters; the 30-gm. level five litters and three resorptions or 62.5% litters; the 50-gm. level six litters and no resorptions or 100% litters. Reference to figure 1 shows that 37.5% litters corresponds to 0.59 mg. of alpha-tocopherol. Since this amount was present in 20 gm., this wheat contained 2.95 mg. per 100 gm.

Calculation of the corresponding results from the 30- and 50-gm. amounts gives 3.23 and 4.00 mg., respectively, of alpha-tocopherol per 100 gm. The weighted average of these results gives 3.34 mg. of alpha-tocopherol per 100 gm. of wheat. It will be noted that there is considerable variation in the individual results for the different feeding levels, which was true in practically all of the samples. However, the averaging of the results in this manner has the advantage of increasing both the number of observations on which the final result is based and presumably the reliability of the data. The values under the column entitled "Alpha-tocopherol per 100-gm. sample" are given to the first decimal only.

The figures given under the column entitled "Feedstuff necessary to produce 4 litters from 5 pregnancies", were calculated from the estimated alpha-tocopherol content of the samples and are included to show the relation of these figures to published results of vitamin E rat assays which are usually reported in terms of litters and resorptions produced.

The figures in table 2 giving the vitamin E content of the wheat samples show that the Ohio samples were higher in each

case than that of the corresponding Arlington farm sample. This appears to indicate a significant difference in vitamin E content due to location in which the wheat was grown. The Fultz variety showed the greatest difference with a value of 3.0 mg. for the sample grown on the Arlington Farm and 5.4 mg. for the Ohio sample.

TABLE 2

Results of vitamin E assays showing estimated alpha-tocopherol content of certain feedstuffs.

VARIETY OR SPECIES	ALPHA-TOCOPHEROL PER 100-GM. SAMPLE	FEEDSTUFF NECESSARY TO PRODUCE 4 LITTERS FROM 5 PREGNANCIES	VARIETY OR SPECIES	ALPHA-TOCOPHEROL PER 100-GM. SAMPLE	FEEDSTUFF NECESSARY TO PRODUCE 4 LITTERS FROM 5 PREGNANCIES
Comparison of varieties of wheat from two locations					
Arlington Farm B. P. I.			Ohio Agric. Expr. Station		
	mg.	*gm.*		*mg.*	*gm.*
Nittany	2.3	54	Nittany	3.5	35
Trumbul	3.0	41	Trumbul	3.5	35
Forward	4.6	27	—	—	—
Fultz	3.0	41	Fultz	5.4	23
Poole	3.3	37	Poole	3.9	32
Comparison of varieties of corn					
Yellow			White		
Woodburn Yellow Dent	3.0	41	Pride of Saline	3.0	41
Midland Yellow Dent	3.6	34	Dakota White Flint	1.5	82
Golden King	2.6	47	Silver King	2.9	42
Comparison of species of grasses and legumes					
Green grass			Dried grass		
Orchard grass	10.9	11.3	Orchard grass	22.3	5.5
Ky. Bluegrass	15.6	7.9	Ky. Bluegrass	35.5	3.5
White clover	10.0	12.3	White clover[1]	7.1	17.3
Alfalfa	15.2	8.1	Alfalfa[1]	10.4	11.8
Redtop	28.1	4.3	Alfalfa leaf meal	38.3	3.2
			Ky. Bluegrass 1 yr. old	23.7	5.2

[1] Samples cured 72 hours at 60° C., accounting for low vitamin E content.

A comparison of the vitamin E content of the different varieties of both wheat and corn samples shows some noteworthy differences. For example, the Arlington Forward wheat sample with a vitamin E content of 4.6 mg. per 100 gm. was twice as rich as the Arlington Nittany sample with a vitamin E content of 2.3 mg. In the case of the corn samples, the range was from 1.5 mg. for the Dakota White Flint to 3.6 mg. for the Midland Yellow Dent.

Vitamin E content of grasses and legumes

The vitamin E content of samples of grasses and legumes representing five species is given in table 2 also. The samples represent products seeded and grown on experimental plots by the Bureau of Plant Industry, Beltsville, Maryland, with the exception of the alfalfa leaf meal. This sample represents a shipment bought on the market and milled approximately 1 month previous to assay. Calculated values are tabulated as in the case of the grains described previously.

Moisture determinations made on the green grasses gave the following results: orchard grass, 70.6%; Ky. bluegrass, 66.6%; white clover, 85.2%; alfalfa, 79.2%; redtop, 75.5%. The other samples are air dry with a moisture content of 10% or less.

The green orchard grass and white clover were not fed at sufficiently low levels to permit resorptions. Consequently, the figures given in the table probably represent a lower vitamin E content than is actually the case.

The samples of orchard and Kentucky bluegrass were dried in an oven at approximately 60° C. for 24 hours. The white clover and alfalfa were dried under the same conditions for 72 hours. It is evident that the longer drying period was sufficiently severe to cause considerable loss in the vitamin E content of the grass. For example, calculation of the alpha-tocopherol content of the green alfalfa to a 10% moisture basis gives a value of 65.8 mg. of alpha-tocopherol per 100 gm., whereas the dried material showed only 10.4 mg. per 100 gm.

DISCUSSION

It is, of course, obvious that the limited number of grass samples does not justify the conclusion that any one species is superior as a source of vitamin E. However, variation in vitamin E content indicated in the assays reported here suggests that variety and species, possibly in conjunction with environmental factors as shown by the results on wheat, are responsible for differences in potency.

The slight advantage of wheat over corn as a source of vitamin E as indicated by average figures for all the grain assays is within expected limits. The relative vitamin E content of the grass samples appears to be high even when compared with assays reported by others. Evans and Burr ('27) reported positive results when feeding as little as 0.6 gm. of alfalfa-leaf meal but this quantity was included in the animals' diet from birth and the total grass fed did not show the high vitamin E content indicated by these assays. These same authors showed lettuce-leaf powder to be a potent source of vitamin E but even these results did not indicate that fresh lettuce is as good a source of vitamin E as, for example, the green alfalfa or redtop reported here.

It is evident from the high vitamin E content of these grasses that the vitamin intake of an animal allowed to graze for a full day would amount to a relatively high quantity. For example, assuming that a 1000 pound cow will consume 100 pounds of grass during a 24-hour period of grazing, the alpha-tocopherol intake would be approximately 7.1 gm. on the basis of the assay figures for Kentucky bluegrass and 12.7 gm. for redtop. This would be equivalent in vitamin E content to 4 to 7 pounds of a relatively high grade wheat germ oil.

SUMMARY

A modified biological assay method for vitamin E is outlined in which alpha-tocopherol is made the basis of estimation.

Experiments are described which show the relative effectiveness of vitamin E supplements fed at different levels to female rats at various stages of the gestation period.

The vitamin E content of five varieties of wheat was estimated to range from 2.3 mg. to 5.4 mg. of equivalent alpha-tocopherol per 100 gm. of grain. The range for six varieties of corn was 1.5 mg. to 3.6 mg. Five species of dried and green grasses and legumes have a range of 7.1 mg. to 28.1 mg.

The vitamin E content of the grasses used for assay is found to be relatively higher than reported by other workers.

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PROCEEDINGS
OF THE NINTH ANNUAL MEETING
OF THE AMERICAN INSTITUTE OF NUTRITION

BOSTON, APRIL 1 AND 2, 1942

The ninth annual meeting of the American Institute of Nutrition was held in Boston at the Lenox and Copley-Plaza Hotels on April 1 and 2, 1942.

COUNCIL MEETING

The Council met at the Hotel Lenox, Tuesday, April 1st, at 4 to 6:30 P.M. and again at 7:30 to 11 P.M. All members were present. Formal actions of the Council are reported in the minutes of the business meetings.

SCIENTIFIC SESSIONS

The usual program on Wednesday began at 9 A.M. at the Copley-Plaza with President Hogan presiding. All but one of the scheduled papers were given. The afternoon session, with Vice-President Maynard presiding, began at 2 P.M. with all papers given. A second scientific session was held in the Hotel Lenox on Thursday from 2 until 5:30 P.M., with Doctors Smith and Sebrell presiding. The abstracts of all papers have been published in the Federation Proceedings, vol. 1, no. 1, part 2, pp. 187–193 (March 16) 1942.

BUSINESS SESSION

Two business meetings were called on Wednesday, April 1st, by President Hogan, one at 11:15 A.M., and the second at 5 P.M. The reading of the minutes of the previous meeting was omitted inasmuch as they had been published in the Journal.

The Treasurer's report was read by Doctor Sebrell. The auditors, Doctors Stiebeling and Jones reported that the accounts were in order. It was moved and seconded that the following recommendations of the Council regarding financial matters be approved: (1) that the dues for 1942–1943 be fixed at $2.00; (2) that the Treasurer invest $500.00 of the Institute funds in a Defense Bond if this be possible; (3) that $.05 per member be paid by the Treasurer for the protection of biological research; and (4) that $25.00 each be allotted to the Secretary and Treasurer for extra secretarial assistance. It was moved and carried that any member who cannot pay dues because of war service should be suspended from membership without prejudice for the duration, and be reinstated if he desires at the end of the war. The Council recommended that the following be elected to membership:

Eliot F. Beach	M. C. Kik	George R. Sharpless
Harold Blumberg	Ruth M. Leverton	Frederick J. Stare
Hugh R. Butt	Cosmo G. Mackenzie	E. L. Robert Stokstad
Jerome W. Conn	Daniel Melnick	Chester D. Tolle
Gladys A. Emerson	Carey D. Miller	Klaus R. W. Unna
A. Edsten Hansen	Luther R. Richardson	

This recommendation was adopted and the candidates were duly elected. The Council recommended the reinstatement of Harry D. Sabotka which recommendation was adopted.

The tellers, J. M. Orten, A. White, H. C. Trimble, W. E. Anderson and A. D. Holmes reported that the following were elected to the offices named:

President	L. A. Maynard
Vice-President	H. B. Lewis
Secretary	A. H. Smith
Treasurer	W. H. Sebrell, Jr.
Councillor	T. H. Jukes
Editorial Board	R. M. Bethke F. A. Hitchcock E. M. Nelson

The changes in the By-Laws recommended last year by the Council were ratified.

The President announced the make-up of the Committee of Judges for the 1942 Mead Johnson Award as follows: R. M. Bethke, G. O. Burr, H. A. Mattill, E. M. Nelson, and C. A. Elvehjem, Chairman. He announced that the award would be made at 4:30 P.M. The President also appointed the following members to the Nominating Committee for 1943: W. H. Chambers, Chairman, H. J. Almquist, N. B. Guerrant, C. G. King and H. A. Mattill.

The Editor of the Journal, Dr. G. R. Cowgill, commented briefly on the year's work, a detailed account of which appears in a following section.

The Committee on changes in By-Laws (Grace Macleod, L. A. Maynard, A. H. Smith, Chairman) reported three suggested revisions which were approved by the Council and which will be sent to the members for consideration before the meeting in 1943.

Dr. A. H. Smith reported on the work of the American Editor of Nutrition Abstracts and Reviews from March to September, 1941, and read Dr. H. H. Mitchell's report on the work from September, 1941, to April, 1942. Doctor Mitchell's appointment as American Editor was confirmed by vote of the members.

Dr. A. J. Carlson set forth the need for financial support by Biological Abstracts. It was moved and seconded that it be recommended to the Council to approve an assessment of $.50 per member annually until further notice; the motion was lost.

The report of the Committee on Vitamin Nomenclature was given by Dr. E. M. Nelson. Attention was called to the suggestion of the term "niacin" and "niacin amide" for "nicotinic acid" and its amide and there was some discussion regarding the dropping of the final "e" in "niacin." The report was adopted.

The recommendation of the Council that the American Association of Immunologists be admitted to the Federation was approved by the members.

A vote of thanks was extended to the Local Committee for the arrangements made for the Institute meetings.

MEAD JOHNSON AWARD

The presentation of the Mead Johnson Award for 1942 was made by President Hogan on behalf of the Institute at 4:30 P.M., Wednesday. The Award was presented to Dr. G. R. Cowgill for "his fundamental contributions through experimental research on the B-vitamins, his leadership in interpreting results of research and his influence in promoting advances in this field of knowledge." Doctor Cowgill, in responding, sketched briefly some of the changes which have occurred in vitamin research over the period covered by his own studies.

EDITORIAL BOARD

During the year 1941 volumes 21 and 22 of The Journal of Nutrition were published; they contained 116 papers. There were submitted for publication during this year 196 papers; 94 were rejected and 9 were withdrawn by the authors; decision still remains to be made on various other papers. The average number of articles printed per issue in volumes 21 and 22 proved to be 9.8. Calculation showed that the average number of pages per article was 11.1. From this it will be seen that the policy of using the limited space in the Journal to cover an average of ten articles per issue is being successfully followed.

The records show that the number of articles continues to exhibit somewhat a seasonal character. For this reason it is not possible to secure equally prompt publication of all articles submitted at all seasons of the year.

Solution of the problem of enlarging the Journal is bound up with that of securing additional income.

During the dinner hour on April 1, 1942, a meeting of the Editorial Board was held at the University Club. Editorial problems and policies were discussed.

Respectfully submitted,

ARTHUR H. SMITH, *Secretary,*
American Institute of Nutrition

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